이학박사학위논문

centrobin의 미세소관 안정화 기능 조절기전 연구

Regulation of the microtubule stabilizing activity of centrobin

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ABSTACT

Regulation of the microtubule stabilizing activity of centrobin

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Microtubules are dynamic cytoskeleton structures in which assembly and disassembly of tubulin dimers occur repeatedly. Microtubule dynamics is regulated by diverse microtubule-associated proteins (MAPs), which are mostly involved in stabilizing or destabilizing microtubules. Centrobin was originally identified as a daughter centriole-specific protein that is required for centriole duplication, but a range of evidences suggest that centrobin is involved in microtubule stabilization. In addition, it has been reported that centrobin is a substrate of two different kinases, NEK2 and PLK1. While the phosphorylation of centrobin by PLK1 is important for the bipolar spindle formation, it has not revealed clearly about the function of the phosphorylation by NEK2. Therefore, in this study, I intended to reveal the function of centrobin regulated by NEK2 phosphorylation and found several important biological phenomena.

Firstly, I found that cytoplasmic centrobin exists as small granule and it is associated with the microtubule network in interphase cells. Furthermore, I also found that the cytoplasmic centrobin forms elongated and large particles along with stable microtubules. These results support that centrobin is involved in the microtubule stabilization.

Secondly, I found that centrobin-depleted cells show disruption of microtubule network and defects in cell spreading, migration and proliferation. On the other hand, depletion of NEK2 that is a kinase for centrobin shows a significant enhancement in microtubule network as well as cell spreading, migration and proliferation. Furthermore, I found that the microtubule stability in interphase cells decreased by centrobin depletion, but significantly increased by NEK2 depletion. These results suggest that centrobin affects the cellular activities in interphase through the microtubule stabilization and NEK2 may negatively regulate the function of centrobin.

Thirdly, I found that four sites of centrobin, T35, S36, S41 and S45 are essential for phosphorylation by NEK2 through a series of in vitro and in vivo kinase assay. The cells stably expressing phospho-resistant centrobin against NEK2 showed significant increase in microtubule stability, cell spreading and migration. However, I observed that the phospho-resistant centrobin against PLK1 had little effect on those phenotypes. These results indicate that the function of centrobin in interphase cells is specifically regulated by NEK2 phosphorylation.

Taken together, the results show that NEK2 regulates the cellular microtubule stability through centrobin phosphorylation and this affects the cell

spreading, migration and proliferation. From the results, I suggest that centrobin is a new type of microtubule-associated protein and NEK2 phosphorylation of centrobin is an important pathway that regulates the microtubule stability of interphase cells.

Keywords:

Microtubule stabilization, NEK2, centrobin, phosphorylation, cell spreading, cell migration

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BACKGROUND

1. Microtubules

Cell cytoskeletons that support physical structure of most vertebrate cells are composed of actin filaments, intermediated filaments and microtubules. Especially, coordination of actin filaments and microtubules are known to be important for diverse cellular process such as cell morphogenesis and migration (Rodriguez et al., 2003; Etienne-Manneville, 2004) (Figure1). Both of them have similar properties in that they have dynamic structure repeating polymerization and depolymerization, and are regulated by Rho signaling pathway (Etienne-Manneville and Hall, 2002). However, whereas actin filaments mainly affect the protrusion and contractility at the cell periphery, microtubules contribute cell polarity near the microtubule-organizing center (MTOC) (Etienne-Manneville, 2004).

1.1. Structure and dynamics

Microtubule has cylindrical structure with 25 nm diameter formed by lateral binding of 12-15 protofilaments. Each protofilament is composed of alternative arrangement of α-/β-tubulin by non-covalent bonds. α-tubulin faces the minus-end of microtubules and β-tubulin faces the plus-end (Figure 2A). Association or dissociation of tubulin heterodimers can occur at both ends of microtubules. However, because the minus-ends of microtubules are usually anchored to the nucleating factors such as γ-

tubulin complex, microtubule dynamics is more active at the plus-ends (Kline-Smith and Walczak, 2004; Honore et al., 2005). GTP-hydrolysis is a major source of power of microtubule dynamics. Both of α- and β-tubulin bind to GTP, and GTP-tubulin dimers can polymerize into microtubules. But GTP bound to β-tubulin is hydrolyzed into GDP after polymerization. The resulting GDP-β-tubulins make the structure of microtubule unstable and induce depolymerization (Howard and Hyman, 2003).

The microtubule dynamics is explained as "dynamic instability" in which microtubules exist in a slow growth state or a rapid shrinkage state, and undergo sudden transitions between those two states (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997). Transition from growth to shrinkage is called catastrophe and the opposite is called rescue (Figure 2B). Microtubule dynamics also can appear as treadmilling. It indicates a phenomenon that microtubule seems to move linearly as a result of polymerization at one end and depolymerization at the other end. So, tubulin flux continuously occurs within microtubule in treadmilling (Rodionov and Borisy, 1997). In animal cells, because most of minus-ends of microtubules are stable, treadmilling occurs at very low frequency (Keating et al., 1997).

1.2. Functions

In interphase cells, microtubules form complex network with MTOC as the center. It primarily regulates the physical activity of cells such as cell morphogenesis and migration. Polarized stabilization of microtubules to the leading edge is important for the efficient cell migration (Bartolini and Gundersen, 2010). Besides, a lot of proteins within cells move along the microtubule network by direct or indirect

interaction with motor proteins such as dyneins or kinesins. For example, about one-third of mitogen-activated protein kinase (MAPK), which is one of the important molecules that are involved in various intracellular signaling, is associated with microtubules by interaction with kinesin (Reszaka et al., 1995; Nagata et al., 1998). This indicates that diverse cellular activities such as proliferation, gene expression, differentiation, survival and apopotosis can be affected by microtubule network. Intracellular transport of vesicles or organelles to specific site is also known to be mediated by microtubule network (Muresan and Muresan, 2012).

In most eukaryotic cells, microtubules form bipolar spindle extended from two opposing spindle poles and function in segregation of duplicated chromosome into two daughter cells. Minus-ends of microtubules face each spindle pole, while plus-ends face metaphase plate. Mitotic spindle is composed of three subpopulations, kinetochore microtubules, interpolar microtubules and astral microtubules (Kline-Smith and Walczak, 2004; O'Connell and Khodjakov, 2007). Kinetochore microtubules are also called as k-fiber. It is attached to kinetochore and stabilized. CENP-E, a kinetochore-associated kinesin, is known to play a important role in the stable attachment of kinetochore and k-fiber (Schaar et al., 1997; Wood et al., 1997). Interpolar microtubules are overlapped with microtubules extended from opposite spindle pole in spindle midzone. Antiparallel microtubule overlaps are important for spindle bipolarity and stability. Microtubule-associated proteins such as PRC-1 and motor proteins have been reported to engage in crosslinking of overlapped microtubules (Bieling et al., 2010; Subramanian et al., 2010; Walczak and Shaw, 2010). Astral microtubules indicate short microtubules of radial array around spindle

pole. They contribute spindle pole positioning and spindle alignment through cell cortex attachment (O'Connell and Khodjakov, 2007).

1.3. Modification and stabilization

Post-translational modifications have critical effects on the properties and functions of microtubules (Westermann and Weber, 2003). They mostly occur on the stable microtubules, and affect the structure and stability through the recruitment of microtubule-relative proteins such as plus-end tracking proteins, motors and severing proteins. Well–known modifications are detyrosination, glutamylation, glycylation and acetylation (Hammond et al., 2008). Detyrosination indicates the detachment of tyrosine from the C-terminal of α-tubulin. It is not known which carboxypeptidase is involved in this process, but re-tyrosination to C-terminal glutamate was reported to occur by tubulin tyrosin ligase (TTL) (Westermann and Weber, 2003). Detyrosination affects the recruitment of plus-end tracking proteins such as CLIP-170 (Peris et al., 2006). Glutamylation and glycylation indicate the attachment of several additional glutamate or glycine to C-terminal glutamate residues of α- or β-tubulin. They are known to be important for the formation and maintenance of the core structures of cilia and flagella called axonemes (Duan and Gorovsky, 2002; Redeker et al., 2005). Acetylation occurs on lysine40 of α-tubulin inside of microtubules. The enzyme that is involved in acetylation has not reported but HDAC6, Sirt2 are known to engage in deacetylation (Hubbert et al., 2002; North et al., 2003). Acetylation occurs on most of stable microtubules, and influences the affinity of microtubule-associated proteins for microtubules (Hammond et al., 2008).

2. Microtubule-related proteins

Many proteins are involved in the regulation of microtubule dynamics. They include motor proteins and non-motor microtubule-associated proteins (MAPs) (Honore et al., 2005) (Figure 3). Motor proteins can be divided into dynein and kinesin family proteins (Muresan and Muresan, 2012). MAPs can be largely divided into structural MAPs and plus-end tracking proteins (+TIPs). The MAPs interact with microtubules directly and stabilize or destabilize it. Besides that, stathmin, a ubiquitous cytosolic protein is known to depolymerize microtubules (Cassimeris, 1999). Microtubule-severing enzymes including katanin and spastin also depolymerize microtubules through its severing activity (Roll-Mecak and McNally, 2010). Patronin was recently reported to bind to the minus-end of microtubules and protect it (Goodwin and Vale, 2010).

2.1. Structural MAPs

Structural MAPs bind along the microtubule lattice and stabilize it. MAP1A, MAP1B, MAP2, MAP4 and Tau belong to this category. MAP1A and MAP1B are predominantly expressed in the dendrites and axons of neuronal cells (Pedrotti et al., 1996; Chien et al., 2005). MAP2, MAP4 and Tau form a family with a conserved carboxy-terminal domain containing microtubule binding repeats. While MAP2 and Tau are mainly found in neuronal cells, MAP4 was reported to be found both in

neuronal and non-neuronal cells (Dehmelt and Halpain, 2005).

2.2. Plus-end tracking proteins

+TIPs are targeted to the growing plus-ends of microtubules through the direct binding with microtubules or interaction with another +TIPs, and affect microtubule dynamics (Akhmanova and Steinmetz, 2008). Some +TIPs are recruited to plus-tips of depolymerizing microtubules or stable microtubules, but most +TIPs are accumulated to the plus-tips of growing microtubules, indicating microtubule dynamics also influences the localization of +TIPs. Well-known +TIPs are EB 1,2,3; CLIP 170/115; CLASP 1/2; $p150^{glued}$; APC; Dis1/TOG family and kinesin 13 family. EB1 is known to enhance microtubule stability through the interaction with APC and mDia or $p150^{glued}$ (Ligon et al., 2003; Wen et al., 2004). Dis/TOG family proteins increase microtubule growth rate by addition of tubulin to the plus-tips of microtubules, or to inhibit catastrophe by suppression of XKCM1, a microtubule depolymerizing factor (Gard and Kirschner, 1987; Tournebize et al., 2000). CLIP family proteins have been reported to contribute microtubule stability by enhancing rescue. CLASP 1/2, the CLIP-associated proteins, also contribute microtubule stability by inhibiting growth and shrinkage (Galjart, 2005). Kinesin-13 family proteins have microtubule depolymerizing activity at the plus-tips. They affect microtubule dynamics in mitotic and interphase cells by stimulating catastrophe or inhibiting rescue (Walczak et al., 1996; Mennella et al., 2005).

2.3. Motor proteins

Microtubule motor proteins move along the microtubules and transport cargoes by hydrolyzing ATP (Gennerich and Vale, 2009). But, besides the function of intracellular transport, they are known to have diverse functions such as force generation for ciliary beating and remodeling of microtubule cytoskeleton (Muresan and Muresan, 2012). Microtubule motor proteins are composed of dyneins and kinesins. While dyneins show minus-end-directed movement, kinesins show plus-end-directed movement except the kinesin-14 family (Gennerich and Vale, 2009). Diverse kinesins have been reported to be involved in the microtubule dynamic. For example, the kinesin-5, kinesin-8, kinesin-13 and kinesin-14 depolymerize microtubules by promoting disassembly or shrinkage (Ferenz et al., 2010; Howard and Hyman, 2007; Peterman and Scholey, 2009). On the other hand, the CENP-E, which is a member of kinesin-7 family, is known to promote microtubule elongation and functions in chromosome congression (McEwen et al., 2001).

3. Cell spreading, migration

Cell spreading and migration are closely connected with each other and share common regulatory molecular mechanisms (Huveneers and Danen, 2009). The interaction between the cell and extracellular matrix (ECM) is critical for the diverse cellular behavior. Integrins are key transmembrane receptors that mediate diverse signaling between the inside and outside of the cell. They detect subtle change in physical interactions of the cell and ECM, and induce the cytoskeleton remodeling

(Harburger and Calderwood, 2009). Src family kinases (SFKs) localize at the cell adhesion sites and activate Rho family GTPases, including RhoA, Rac1, Cdc42, through the control of the guanine-exchanging factors (GEFs) and the GTPase-activating proteins (GAPs) (Mitra and Schlaepfer, 2006; Tomar and Schlaepfer, 2009). Several effector proteins act on these Rho-GTPase and engage in the regulation of actin or microtubule dynamics, which affect the cell spreading and migration (Huveneers and Danen, 2009).

3.1. Cell spreading

During the early stage of cell spreading, focal adhesion kinase (FAK) is autophosphorylated at tyrosine 397 by the integrin-mediated interaction of the cell and ECM (Mitra et al., 2005). Then, Src binds this site through the Src-homology 2 (SH2) domain and phosphorylates other tyrosine residues of FAK (Mitra and Schlaepfer, 2006). This FAK-Src complex activates Rac1 or Cdc42 through the phosphorylation of p130Cas or paxillin (O'Neill et al., 2000; Brown et al., 2005). The activated Rac1 and Cdc42 are known to induce the formation of lamellipodia and filopodia, respectively (Jaffe and Hall, 2005). At the same time, the FAK-Src complex suppresses the RhoA activity through the p190RhoGAP phosphorylation, which inhibits the actomyosin contractility and the formation of stress fibers (Arthur et al., 2000). The induction of membrane protrusion and the inhibition of cytoskeletal contractility are thought to facilitate cell spreading. On the contrary, the activity of Rac1 and Cdc42 decreases and that of RhoA increases during the later stage of cell spreading, which stimulates the formation of stress fibers and the maturation of focal

adhesion (Tomar and Schlaepfer, 2009). These coordinations of Rac1, Cdc42 and RhoA activity contribute the efficient cell spreading by regulating the actin- and microtubule-cytoskeleton spatiotemporally.

3.2. Cell migration

Migrating cells are polarized toward the direction of migration. They display a polarized cytoskeleton and asymmetric distribution of signaling molecules. Lamellipodia and filopodia are formed at the leading edge, and membrane retraction occurs at the trailing edge of migrating cells. MTOC and golgi apparatus are oriented to the leading edge, and some microtubules are captured and stabilized at the cell cortex of leading edge (Vicente-Manzanares et al., 2005). Rho family GTPases and its effector proteins are also important for the coordinated regulation of these processes (Figure 4). Rac1 and Cdc42 localize at the leading edge of migrating cells and engage in the formation of lamellipodia and filopodia through the regulation of actin and microtubule dynamics. Cdc42 also localizes at the golgi apparatus and plays a role in the formation of cell polarity (Watanabe et al., 2005). RhoA seems to engage in the retraction of trailing edge through the regulation of actomyosin contractility (Worthylake et al., 2001). RhoA was also reported to be involved in the microtubule stabilization at the leading edge through the mDia-APC-EB1 pathway (Palazzo et al., 2004).

4. Centrosome

Centrosome is a cytoplasmic small organelle that serves as a microtubule organizing center (MTOC) during interphase and a spindle pole during mitosis. The function of MTOC is important for the cell polarity and migration. And, the function of spindle pole is important for the exact separation of genomic material into two daughter cells. Therefore, centrosome is associated with cancer and the abnormality in centrosome number is often found in many types of tumor (Bakhoum and Compton, 2009).

4.1. Structure and functions

Centrosome is composed of two centrioles arranged perpendicularly with defined length and diameter (Figure 5). The proximal part of each centriole shows a structure of nine microtubule triplets while distal part of that shows nine microtubule doublets. Of the two centrioles, the older is called as mother centriole and the newer is called as daughter centriole. They have differences in structure and function. The mother centriole is longer and more matured, and has two sets of nine appendages at its distal end (Bornens, 2012). Daughter centriole is newly synthesized based on the mother centriole, but little is known about the function of daughter centriole. Centrobin is the only protein that has been reported to show daughter centriole-specific localization (Zou et al., 2005; Jeong et al., 2007). The two centrioles are surrounded by pericentriolar material (PCM). There are more than a hundred of proteins in PCM including γ-tubulin which is important for microtubule nucleation (Rusan and Rogers, 2009).

4.2. Cycle

The centrosome cycle is coupled with the cell cycle. Centrosome is duplicated once per one cell cycle. Like the chromosomes, duplication of centrosome occurs during the S-phase, and PCM starts to be accumulated from this moment. As cells go through G2 phase, PCM recruitment increases and its microtubule nucleating activity also increases. In this period, centrosome separation occurs for the formation of bipolar spindle in mitosis. As cells enter mitosis, PCM recruitment increases more and the nucleating activities become the highest at metaphase. As cells exit mitosis, the level of PCM also decreases and two centroles are disengaged (Rusan and Rogers, 2009; Nigg and Stearns, 2011).

5. NEK (NIMA-related kinase) family proteins

NIMA-related kinases are a family of serine/threonine kinases which are involved in the regulation of cell cycle progression (O'Connell et al., 2003). NIMA (Never In Mitosis A) kinase of Aspergillus nidulans is known to be important for the mitotic entry and spindle formation (Lu and Hunter, 1995). Human has 11 NEK family proteins, NEK1 to NEK11. They have about 40% of homology with NIMA in kinase domain. Among them, NEK2, NEK6, NEK7 and NEK9 have been studied well and known to be related with the mitotic progression (O'regan et al., 2007).

5.1. NEK2

NEK2 is a NEK family member that has highest homology with NIMA (about 44%) in kinase domain. It is composed of N-terminal kinase domain and C-terminal regulatory domain (Figure 6A). There are three alternative splice variants, NEK2A, NEK2B and NEK2C (Fardilha, 2004). NEK2A and NEK2B are expressed in diverse cell line although the expression level of NEK2A is generally higher than NEK2B. However, it was reported only NEK2B is expressed in oocytes and early stage of embryos, indicating its special role in early embryogenesis (Uto et al., 1999). The expression level of NEK2 in culture cell is low in G1 phase and high in S/G2 phase (Schultz et al., 1994). Upon entering mitosis, NEK2A is degraded fast, but the level of NEK2B remains the same. In concordance with the expression level, the kinase activity of NEK2 is also low in G1 and high in S/G2 (Fry et al., 1995). The best characterized function of NEK2 is to induce the centrosome separation at G2/M transition (Fry et al., 1998a; O'Regan et al., 2007) (Figure 6B). C-NAP1 and Rootletin, the intercentriolar linker proteins, are phosphorylated by NEK2 and dissociated from centrosome (Fry et al., 1998b; Bahe et al., 2005). As a result, centrosome separation is induced, and this contributes the formation of bipolar spindle in mitosis. NEK2 is also thought to function in microtubule organization through its two substrate, Nlp and centrobin, which were reported to be required for the microtubule nucleation and stabilization, respectively (Rapley et al., 2005; Jeong et al., 2007). Besides, NEK2 has been reported to be involved in the chromatin condensation in meiotic spermatogenesis (Di Agostino et al., 2002), or mitotic checkpoint through the interaction with Hec1 and Mad1 (Chen et al., 2002; Lou et al., 2004).

6. Centrobin

Centrobin is originally identified by yeast two-hybrid screening with BRCA2 or NEK2 as bait. It was revealed that human centrobin is a 903 amino acid protein with coiled-coil region at its center. Centrobin is detected at the centrosome in both of interphase and mitosis, and shows preferential localization of daughter centriole (Zou et al., 2005; Jeong et al., 2007). It has been also reported that centrobin is required for the centrosome duplication, and affects centriole elongation and stability through the interaction with tubulin (Gudi et al., 2011). But, there are two opposite results about the function of centrobin. While one report observed no gross abnormalities in microtubule nucleation and organization when centrobin was depleted, another report showed significant reduction of microtubule organizing activity and cell shrinkage by centrobin depletion (Zou et al., 2005; Jeong et al., 2007). In addition, a biochemical study showed that centrobin is associated with microtubules in vivo and in vitro, and had microtubule polymerizing activity. The study also showed PLK1 affects the microtubule polymerizing activity of centrobin, and suggested centrobin may regulate the spindle assembly through the PLK1 phosphorylation (Lee et al., 2010).

Figure 1. Organization of actin and microtubule cytoskeleton in cellular morphogenesis.

Two regions can be distinguished in cellular morphogenesis: an actin-rich region (red) comprising actin bundles organized into filopodia, a dense actin meshwork forming a ruffling lamellipodia and only a few pioneer microtubules; and a microtubule-rich region (green), where microtubules radiate from the centrosome (dark green star) with their plus-ends directed towards the plasma membrane and where actin structures are essentially limited to stress fibers (thick red lines), anchored to the substrate via focal adhesions (blue dots). Adapted from Etienne-Manneville. (2004).

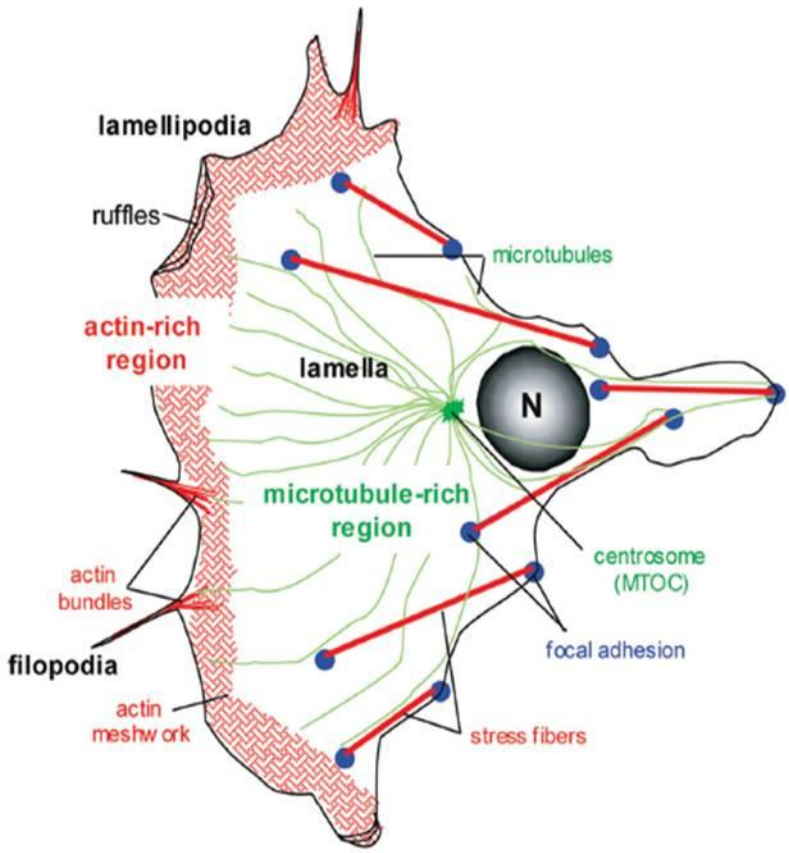
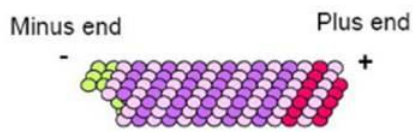

lamellipodia
ruffles
microtubules
actin-rich
region
lamella
N
microtubule-rich
region
centrosome
(MTOC)
actin
bundles
filopodia
focal adhesion
actin
meshwork
stress fibers

Figure 2. Microtubule structure and dynamic instability.

(A) A MT is a polarized polymer of α-/β-tubulin heterodimers. During polymerization the GTP bound to the β tubulin subunit is hydrolyzed (GTP is also bound to the α-subunit, but this is not exchangeable or hydrolyzed). (B) MTs alternate between phases of polymerization and depolymerization at their plus ends. Transitions from shrinkage to growth are known as rescues while the opposite reaction is known as catastrophe. Adapted from Bartolini and Gundersen. (2010).

A.


Minus end
Plus end
-
+

B.

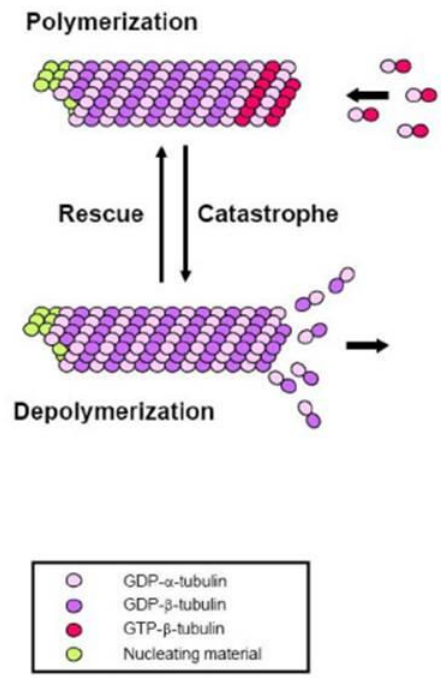
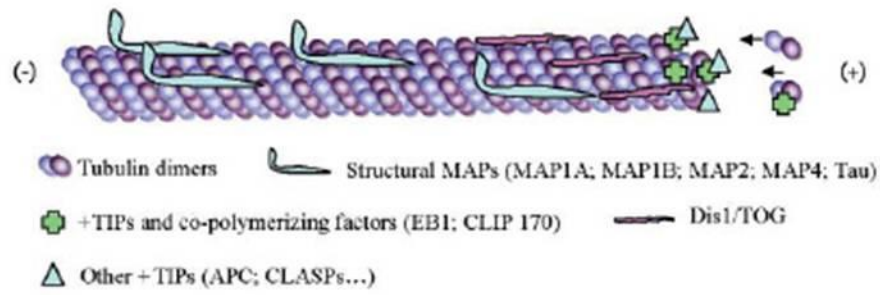

Polymerization
Rescue
Catastrophe
Depolymerization
GDP-α-tubulin
GDP-β-tubulin
GTP-β-tubulin
Nucleating material

Figure 3. Microtubule-related proteins.

(A) A growing MT and various interactions with stabilizing factors. (B) A shortening MT and various interactions with destabilizing factors. (–) and (+) indicate the minus and the plus ends of the MT, respectively. Adapted from Honore et al. (2005).

A.


(-)
(+)
Tubulin dimers
Structural MAPs (MAP1A; MAP1B; MAP2; MAP4; Tau)
+TIPs and co-polymerizing factors (EB1; CLIP 170)
Dis1/TOG
Other +TIPs (APC; CLASPs…)

B.

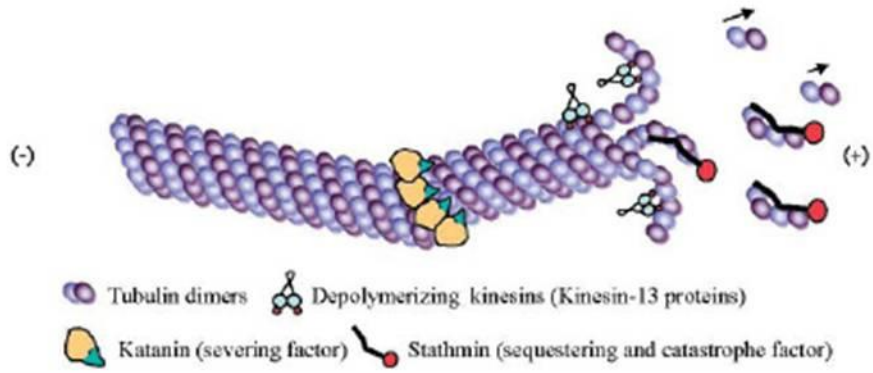
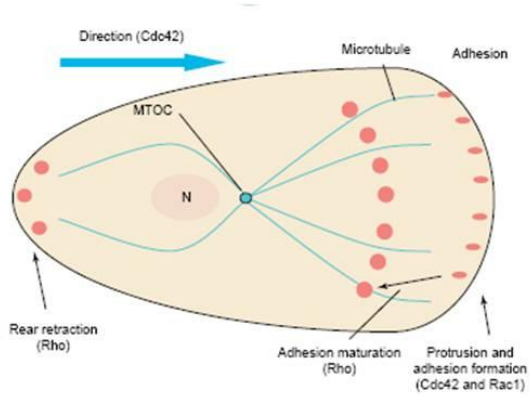

(-)
(+)
Tubulin dimers
Depolymerizing kinesins (Kinesin-13 proteins)
Katanin (severing factor)
Stathmin (sequestering and catastrophe factor)

Figure 4. Rho family GTPases in cell migration.

(A) Cdc42, together with Par proteins and aPKC, is involved in generating cell polarity. At the front of the cell, Rac1 and Cdc42 regulate the formation of ruffles and filopodia, respectively. RhoA is thought to be involved in retraction at the rear of the cell through the regulation of actin–myosin contractility. Activation of RhoA also induces the maturation of focal adhesions behind leading edges. (B) Rho family GTPases regulate microtubules through their effectors (e.g. Par6, IQGAP1, PAK and mDia). These effectors interact with +TIPs (e.g. APC, EB1 and CLIP-170) or modify the activities of +TIPs and MAPs (e.g. APC and stathmin). Adapted from Watanabe et al. (2005).

A.


Direction (Cdc42)
Microtubule
Adhesion
MTOC
N
Rear retraction
(Rho)
Adhesion maturation
(Rho)
Protrusion and
adhesion formation
(Cdc42 and Rac1)

B.

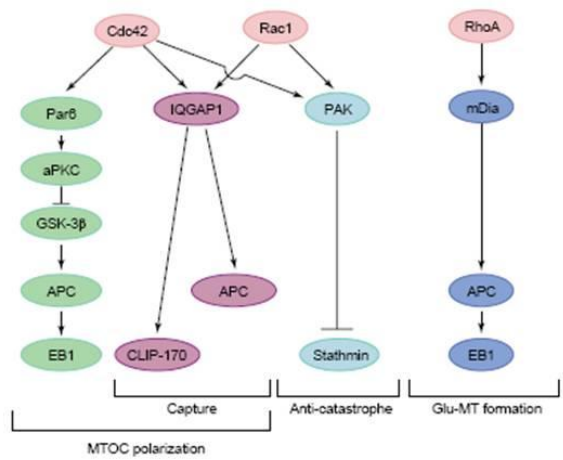

Cdc42
Rac1
RhoA
Par6
IQGAP1
PAK
mDia
aPKC
GSK-3β
APC
APC
APC
EB1
CLIP-170
Stathmin
EB1
Capture
Anti-catastrophe
Glu-MT formation
MTOC polarization

Figure 5. The centrosome of human cells.

Centrosome contains a structurally and functionally asymmetric pair of centrioles, a mother centriole and a daughter centriole. The mother centriole is distinguished by two sets of nine appendages at its distal end, which are required for anchoring microtubules. Adapted from Bornens. (2012).

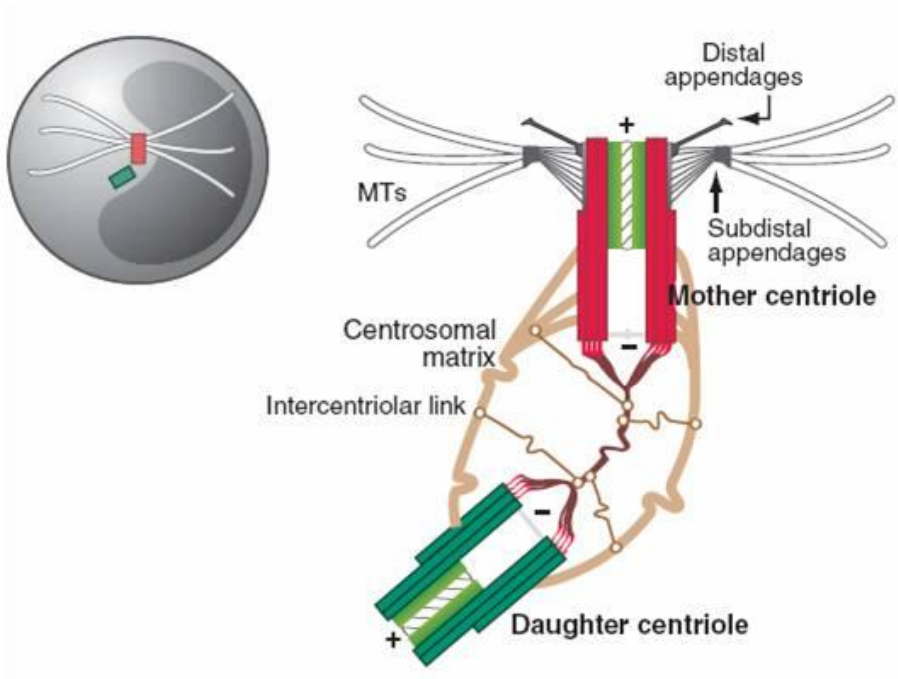
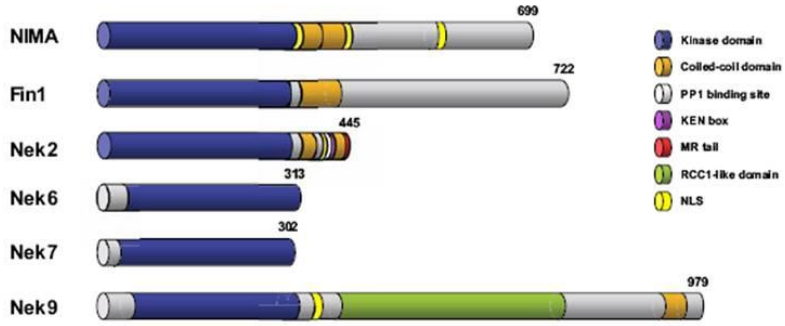

Distal
appendages
+
MTs
Subdistal
appendages
Mother centriole
Centrosomal
matrix
–
Intercentriolar link
–
Daughter centriole
+

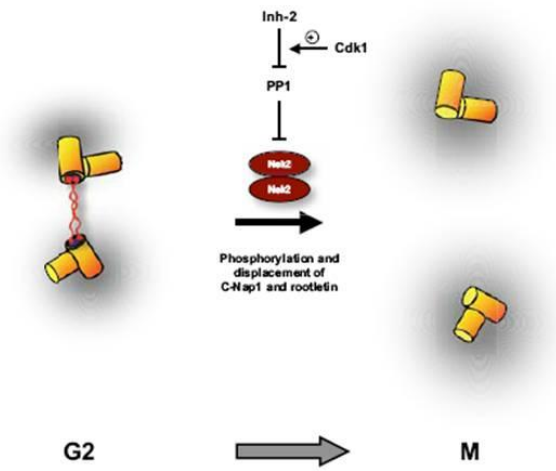
Figure 6. The structure and function of NEK2.

(A) A schematic representation of the two fungal (*Aspergillus* NIMA and *S. pombe* Fin1) and four mammalian (NEK2, NEK6, NEK7 and NEK9) NIMA-related kinases. Among the three splice variants of NEK2, NEK2A is shown here. Numbers represent protein length in amino acids. (B) NEK2 promotes centrosome separation at mitotic onset. Activation of NEK2 leads to phosphorylation and displacement of C-Nap1 and rootletin from the centrosome. The two disconnected pairs of centrioles can then be driven apart to form the two poles of mitotic spindle. Adapted from O'regan et al. (2007).

A.


NIMA
699
Fin1
722
Nek2
445
Nek6
313
Nek7
302
Nek9
979
Kinase domain
Coiled-coil domain
PP1 binding site
KEN box
MR tail
RCC1-like domain
NLS

B.


Inh-2
Cdk1
PP1
Nek2
Nek2
Phosphorylation and
displacement of
C-Nap1 and rootletin
G2
M

PURPOSE

Cell cytoskeletons are important regulators of diverse cellular activities such as cell morphogenesis, migration, intracellular signaling and organelle transport. These cellular activities are essential for the many biological processes. For example, cell migration is closely related with development, angiogenesis, wound healing and cancer metastasis. So, studying the dynamics of cytoskeleton is very helpful to understand living organism.

Centrobin is known as a subustrate of NEK2. Centrobin and NEK2 are known to localize at the centrosome, and to be involved in centrosome duplication and separation, respectively. But there exist some evidences that both of them are associated with microtubules. Therefore, I intended to investigate if NEK2 and centrobin are involved in the regulation of microtubule dynamics within cells with following questions.

Firstly, what effects do the NEK2 and centrobin have on the microtubules within cells? In spite of several evidences that they are associated with microtubules, it is not clearly known how they act on microtubules within cells. To reveal this, I performed a series knockdown and rescue experiments, and investigated the effects on the cytoplasmic microtubules.

Secondly, what effect do the NEK2 and centrobin have on the cells through the regulation of microtubules? As mentioned previously, microtubules affect the diverse cellular processes. So, I investigated about the effects of NEK2 and centrobin

on cell spreading, migration and proliferation.

Thirdly, is the centrobin phosphorylation by NEK2 important for the regulation of microtubules? To answer this question, I tried to find the phosphorylation site of centrobin, and test whether the phopho-mutant centrobin has specific effects on microtubules within cells.

In conclusion, I intended to study the regulation mechanism of cytoplasmic microtubules and its effects on the cellular processes through the centrobin phosphorylation by NEK2.

INTRODUCTION

Together with actin filaments, microtubules are main components of cell cytoskeleton. In interphase, microtubules form cytoplasmic array around microtubule organizing center (MTOC) to support not only cell structure but also various cellular processes such as cell migration, spreading, intracellular signaling and organelle transport (Honore et al., 2005). In mitosis, microtubules form bipolar spindle to draw chromosomes into spindle poles (Walczak et al., 2010). Spatial and temporal coordination of diverse microtubule associated proteins (MAPs) and regulatory kinase and phosphatase are important for the accurate regulation of microtubule dynamics (Drewes et al., 1998; Cassimeris, 1999).

Interphase cells have two distinct populations of microtubules. While most of interphase microtubules show dynamic instability with short half-lives (< 20 min), some microtubules are more stable and have longer half-lives (> 1 h) (Schulze and Kirschner, 1987). Stable microtubules are characterized by abundance in detyrosinated and acetylated α-tubulin and enhanced resistance to microtubule depolymerizing agents such as nocodazole (Gundersen et al., 1987; Piperno et al., 1987). Diverse MAPs are involved in microtubule stabilization. For example, EB1 promotes microtubule stabilization by affecting the structure of microtubule plus end or interacting with other microtubule plus-end tracking proteins (+TIPs) such as APC,CLASP and CLIP family proteins (Tirnauer et al., 2002; Vaughan, 2005; Galjart, 2010). On the other hand, kinesin-13 family members are known to destabilize

microtubules by stimulating catastrophe or suppressing rescue at the microtubule plus end (Mennella et al., 2005). Classic MAPs, such as MAP1A, MAP1B, MAP2, MAP4, Tau, stabilize microtubule through lateral binding to microtubule and organize microtubule network by microtubule crosslinking (Dhamodharan and Wadsworth, 1995; Nguyen et al., 1997; Bunker et al., 2004).

Microtubule stabilization is closely related with cell migration. Rho family GTPases, such as Rac, Cdc42 and RhoA, play important roles in cell migration through the regulation of microtubule dynamics as well as actin filament (Watanabe et al., 2005; Tomar and Schlaepfer, 2009). RhoA contributes microtubule stabilization at leading edge by regulation of APC and EB1 localization via mDia (Palazzo et al., 2001; Wen et al., 2004). The Rho-mDia pathway is known to be facilitated by the integrin-Fak signaling pathway (Palazzo et al., 2004). Rac1 and Cdc42 participate in the polarized microtubule stabilization by inhibiting the stathmin activity or regulating localization of CLIP-170 and APC at cortical region through its effector proteins (Daub et al., 2001; Wittmann et al., 2004; Fukata et al., 2002; Watanabe et al., 2004). Microtubule stabilization is also related to cell morphogenesis (Bulinski and Gundersen, 1991). In macrophage, CLIP-170, a plus-end targeting protein, was reported to enhance cell spreading by stabilizing microtubules (Binker et al., 2007).

NEK2, a member of NIMA-related kinases, is a serine/threonine kinase implicated in cell cycle control (Schultz et al., 1994; Fry et al., 1995). NEK2 is best-known as a kinase for centrosome separation during the centrosome cycle (Fry et al., 1998a). C-NAP1 and rootletin are NEK2 substrates responsible for centrosome separation prior to M phase (Fry et al., 1998b; Bahe et al., 2005). Recently, additional

substrates of NEK2 have been reported. For example, Nlp, a centrosome protein for microtubule nucleation during M phase, is phosphorylated and activated by PLK1 (Casenghi et al., 2003). NEK2 also phosphorylates Nlp for priming the PLK1 phosphorylation (Rapley et al., 2005). Many reports propose that NEK2 is involved in multiple cellular processes, such as microtubule organization, chromatin condensation, mitotic checkpoint and cytokinesis (O'Regan et al., 2007). In addition, elevated expression of NEK2 was observed in diverse human cancer cell, suggesting that NEK2 may contribute to cancer progression (Wai et al., 2002; Hayward et al., 2004).

It has been also reported that centrobin is a substrate of two mitotic kinases, NEK2 and PLK1, and has a microtubule stabilizing activity (Jeong et al., 2007; Lee et al., 2010). In addition, centrobin was reported to regulate the assembly of functional mitotic spindles (Jeffery et al., 2010).

In this study, I analyzed centrobin, a substrate of NEK2 (Jeong et al., 2007). Centrobin is a daughter centriole-associated protein required for centriole duplication, elongation and stability (Zou et al., 2005; Gudi et al., 2011). Biological functions of centrobin remain elusive, but are suggested as a microtubule stabilizer (Lee et al., 2010). In this dissertation, I determined that centrobin is involved in microtubule stabilization. Furthermore, I revealed that NEK2 specifically phosphorylates centrobin and controls biological functions of centrobin in cell spreading, migration and proliferation.

MATERIALS AND METHODS

Antibodies, transfection and RNA interference

α-tubulin (Sigma), acetylated α-tubulin (Sigma), γ-tubulin (Sigma for mouse or Santa Cruz for goat), HA (Sigma), FLAG (Sigma) and NEK2 (BD bioscience) antibodies were used according to the manufacturer′s instruction. Centrobin and CP110 antibodies were used as previously described (Jeong et al., 2007; Kim et al., 2008). Transient transfection of plasmid DNA was performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer′s instruction. For RNA interference, siRNAs specific to *NEK2* (5′-GGC AAA TTC AGG CGA ATT C-3′), *NEK2*-3′UTR (5′-GCT GTA GTG TTG AAT ACT T-3′) and *centrobin* (5′-GGA TGG TTC TAA GCA TAT C-3′) were purchased from ST Pharm and transfected into the cells using Lipofectamine RNAi max (Invitrogen) according to the manufacturer′s instructions. Non-specific control siRNA (5′-AAG TAG CCG AGC TTC GAT TGC-3′) was also used.

Cell culture and stable cell lines

293T, HeLa and tet-on HeLa cells were cultured in DMEM supplemented with 10% FBS. U2OS cells were culured in McCoy′s 5A media supplemented with 10% FBS. Stable tet-on HeLa cell lines were generated with Lenti-X HT packaging system (Clontech) according to manufacturer′s instruction using pLVX-IRES-Puro vector substituted its original promoter with tet-responsive promoter.

Immunoblot and immunoprecipitation

For immunoblot analysis, protein samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was incubated with a primary antibody for 2 h after blocking with 5% skim milk in 0.1% TBST (Tris-buffered saline (TBS) with 0.1% Triton X-100) for 30 min, and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min after washing three times with 0.1% TBST. And then, the membrane was incubated with the ECL solution after washing three times with 0.1% TBST, and exposed to an X-ray film.

For immunoprecipitation, the cells were lysed with the RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) or the NP40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40) with protease inhibitors for 20 min on ice and centrifuged with 15,000 *g* for 20 min at 4°C. The supernatant was incubated with specific antibodies for 2 h, followed by protein A Sepharose (Amersham Pharmacia) for 2 h at 4°C.

Immunocytochemistry and image processing

For immunochemistry, the cells cultured on coverslip were fixed with cold methanol for 10 min after washing with phosphate-buffered saline (PBS). The fixed cells were blocked with 5% bovine serum albumin (BSA) in 0.1% PBST (PBS with 0.1% Triton X-100), incubated with the primary antibodies for 1 h, washed with 0.1%

PBST three times, and incubated with secondary antibodies for 30 min. And then, the cells were washed three times with 0.1% PBST, incubated with DAPI solution to stain DNA, and the coverslip was mounted on a glass slide. The immunostained cells were observed using fluorescence microscope with a CCD (Qicam Fast 1394; Qimaging) camera and processed with ImagePro 5.0 (Media Cybernetics, Inc.) software.

Nocodazole-resistance assay

Nocodazole-resistance assay was performed as previously described with a slight modification (Khawaja et al., 1988). In brief, the cells cultured on coverslip were treated with 2 mM thymidine for 16 h to synchronize cell cycle and incubated with 2 μM nocodazole for the last 30-60 min at 37°C. And then, the cells were rinsed twice in PEM buffer (100 mM PIPES [pH 6.9], 1 mM EGTA, 2 mM $MgCl_2$), incubated 1 min at 37°C with 0.2% Triton X-100 in PEM to remove monomeric tubulin, rinsed again twice in PEM buffer, and fixed with cold methanol. After fixation, the cells were subjected to immunocytochemistry using acetylated α-tubulin antibody. To quantitative analysis, the cells were scored for the presence of more than 10 acetylated microtubules per cell.

Phosphorylation assays

For in vitro kinase assay, NEK2 kinases were prepared from 293T cells transfected with wild-type or kinase-dead NEK2 expression vectors. The cells were lysed with NP40 lysis buffer and subjected to immunoprecipitation with an antibody against the HA tag. The immunoprecipitates were washed twice with lysis buffer and

once with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 g/ml heparin). The centrobin substrates were prepared from bacterially expressed fusion proteins. Kinase reactions were carried out for 30 min at 30°C in kinase buffer supplemented with 5 μM ATP, 1 mM dithiothreitol and 5 μCi [γ-^{32}P]ATP in a total volume of 20 μM. The reactions were stopped by adding 2×SDS sample buffer and heating for 5 min at 95°C. Protein samples were subjected to SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was exposed to a BAS plate or an X-ray film to obtain an autoradiograph image, and then stained with Coomassie brilliant blue solution.

In other to detect the phosphorylation of purified centrobin from the cells, wild-type or phoshpo-resistant centrobin were co-transfected with wild-type or kinase-dead NEK2 into 293T cells. The cells were lysed with RIPA buffer and the ectopic centrobin proteins were purified using anti-FLAG beads. Purified proteins were subjected to SDS-PAGE and transferred onto a PVDF membrane and stained using Pro-Q diamond phosphoprotein blot stain kit according to manufacturer′s instruction (Invitrogen).

Cell migration and cell spreading

For cell migration assay, cells were grown to subconfluency and treated with 10 μg/ml Mitomycin C (Sigma) for 3 h to arrest cell proliferation. And then, a wound track was introduced by scraping the cell monolayer with a yellow pipette tip. After rinsing with PBS, the cells were cultured in growth medium for a further 24 h and the recovery area was measured. For cell spreading assay, cells were treated with

2 mM thymidine for 16 h to synchronize cell cycle, and the cell area was measured. For measurement of the area, phase-contrast images were analysed using ImagePro 5.0 software.

RESULTS

Centrobin is associated with cytoplasmic microtubules

I performed co-immunostaining analysis of centrobin in U2OS cells with antibodies specific for centrobin and centrin. The results showed a strong centrobin signal at the daughter centriole which was weakly stained with the centrin antibody (Figure 7A). When the same fluorescent image was detected longer, I was able to detect the cytoplasmic signals (Figure 7A). I carefully determined whether the cytoplasmic signals of centrobin are specific or not. In fact, the granulated signals of centrobin were detected throughout the cytoplasm (Figure 7B). Furthermore, these granulated signals of centrobin disappeared along with the centrosome signal in centrobin-depleted cells (Figures 7B). These results indicate that centrobin is localized not only at the daughter centriole but also throughout the cytoplasm as granules (Figure 7; Zou et al., 2005; Jeong et al., 2007).

A previous result indicates that centrobin interacts with and stabilizes microtubules (Lee et al., 2010). In accord, the co-immunostaining analysis revealed that the cytoplasmic centrobin is placed in close association with the microtubule network in interphase cells (Figure 8). Furthermore, the cytoplasmic centrobin often forms elongated, large particles along with stable microtubules which are immunostained with the acetylated α-tubulin antibody (Figure 9A). The ectopic centrobin protein also showed a similar subcellular distribution pattern with the endogenous protein forming aggregates in association to the stable microtubules

(Figure 9B). Taken together, these results show that the cytoplasmic centrobin is on the microtubules, especially in associated with stable microtubules. This subcellular distribution is consistent with the hypothesis in which centrobin is involved in the microtubule organization and stabilization.

The NEK2- and centrobin-depleted cells reveal opposite outcomes in cell morphology, proliferation and migration

Centrobin is known as a substrate of NEK2, a member of NIMA-related kinases (Jeong et al., 2007). To investigate the functional relationship between NEK2 and centrobin, I knocked down the endogenous NEK2 and centrobin proteins and compared their phenotypes in HeLa, U2OS and RPE1 cells. The cellular levels of NEK2 and centrobin were effectively reduced within 48 h after transfection of specific siRNAs for *NEK2* (*siNEK2*) and *centrobin* (*siCBN*) in all three cell lines (Figure 10).

As reported previously, the cells are shrunk and eventually dead as soon as centrobin is depleted (Figure 5A; Jeong et al., 2007). On the other hand, the NEK2-depleted cells were rather spread in all three cell lines (Figure 11A). The quantitative analysis showed that the area of the NEK2-depleted cells significantly increased, while that of the centrobin-depleted cells decreased in all the tested cell lines (Figure 11B).

I determined the proliferation activities of the NEK2- and centrobin-depleted cells. The results showed that the NEK2-depleted cells proliferate faster than the control cells (Figure 12). On the other hand, the centrobin-depleted cells proliferate

slower than the control cells, partly due to mitotic arrest and apoptosis (Figure 12; Jeong at al., 2007).

When the NEK2-depleted cells were immunostained with the α-tubulin antibody, I observed the microtubule network developed strongly around the nucleus of HeLa, U2OS and RPE1 cells (Figure 13). The centrobin association with microtubules was more extensive in NEK2-depleted cells than control cells (Figure 14). However, the microtubule network was considerably disorganized in centrobin-depleted cells (Figures 13 and 14). Actin filaments in NEK2- or centrobin-depleted cells were also examined with TRITC-conjugated phalloidin. The results showed that the actin stress fibers increased significantly in NEK2-depleted cells (Figure 15). On the other hand, an abnormal organization of actin cytoskeleton was observed in centrobin-depleted cells (Figure 15). These results suggest that cell motility is enhanced in NEK2-depleted cells whereas the opposite is the case in centrobin-depleted cells.

I determined the cell motility by measuring the distance of cell migration after scraping off a part of the monolayer cells of the NEK2- or centrobin-depleted cells. The results showed that the NEK2-depleted cells migrated faster than the control cells (Figure 16). However, the centrobin-depleted cell did not migrate efficiently (Figure 16). Taken together, I concluded that NEK2 and centrobin depletion result in opposite effects on the cell morphology, proliferation and migration, probably as a result of opposite effects on the cytoskeletal organization of the cells.

The microtubule network is stabilized in NEK2-depleted cells but

destabilized in centrobin-depleted cells

Acetylation makes microtubules stable and, as a result, resistant to destabilizer such as nocodazole (Gundersen et al., 1987; Piperno et al., 1987). I found a significant amount of acetylated microtubules still remained in NEK2-depleted cells after 2 μM nocodazole treatment for 30 min (Figure 17A). On the other hand, a less amount of acetylated microtubules were detected in the centrobin-depleted cells than the control cells (Figure 17A). I quantified the stability of microtubules by measuring the resistance against nocodazole (Khawaja et al., 1988). When the HeLa cells were treated with nocodazole for 0.5 and 1 h, acetylated microtubules were detected in 40% and 20% of the control cells, respectively (Figure 17B). The number of the acetylated microtubule-positive cells increased by two-fold by NEK2 depletion (Figure 17B). On the other hand, the number of nocodazole-resistant cells decreased to a half by centrobin depletion (Figure 17B). Co-depletion of NEK2 and centrobin showed similar nocodazole-resistance with a single depletion of centrobin, suggesting that NEK2 may stabilize microtubules through centrobin (Figures 17A and 17B). I also measured the fluorescent intensity of acetylated microtubules in nocodazole-resistant cells. The results showed that the NEK2-depleted cells include more nocodazole-resistant microtubules than the control whereas the centrobin-depleted cells are the opposite (Figure 17C).

I generated stable cell lines in which the expression of siRNA-resistant NEK2 is induced by doxycycline. Immunoblot analysis confirmed that doxycycline enhanced the ectopic NEK2 levels significantly (Figure 18A). At the same time, a considerable amount of ectopic NEK2 was detected in the stable cells even without

induction (Figure 18A). I performed rescue experiments by transfection of *siNEK2* into the inducible NEK2 stable lines. As shown previously, NEK2 depletion increased the number of cells with nocodazole-resistant acetylated tubulin and the ectopic NEK2 canceled out this phenotype (Figure 18B). However, the kinase-dead NEK2 could not rescue this phenotype, indicating that the kinase activity of NEK2 is required for destabilization of microtubule networks in interphase cells (Figure 18B). I also performed rescue experiments with the centrobin-depleted cells and observed that ectopic centrobin rescued the nocodazole-resistance to the control levels (Figure 19). Collectively, these results suggest that centrobin stabilizes microtubule network but NEK2 disturbs the stability of microtubules.

Determination of specific NEK2 phosphorylation sites of centrobin

In order to test the hypothesis in which NEK2 engages in regulation of cytoplasmic microtubule stability via centrobin phosphorylation, I decided to pinpoint NEK2 phosphorylation sites within the centrobin protein. The previous study revealed that NEK2 specifically phosphorylates 1-193 residues of centrobin (Figure 20A; Jeong et al., 2007). I performed a series of in vitro kinase assays of NEK2 with truncated constructs of the centrobin fusion porteins (GST-CBN), and eventually limited specific NEK2 phosphorylation sites to 30-56 residues of centrobin (Figure 20B). The candidate phosphorylation sites of NEK2 should be distinct from those of PLK1 which are located at 1-29 residues of centrobin (T3, S4, S21 and S22; Lee et al., 2010).

The 30-56 residues of centrobin have seven serine/threonine sites (Figure

21A). I prepared GST-CBN$^{30\text{-}56}$ in which each of serine and threonine residues was substituted with alanines and used them as substrates. The results showed that NEK2 hardly phosphorylated the alanine-substituted mutants at T35, S36, S41 and S45 (Figure 21B). Therefore, these four sites are considered candidate phosphorylation sites for NEK2.

NEK2 phosphorylation of centrobin was examined in 293T cells expressing the ectopic proteins. Immunoblot analysis revealed that the FLAG-tagged centrobin protein (FLAG-CBN) generates a slow- and a fast-migrating band in the presence of ectopic NEK2 (FLAG-NEK2) (Figure 22A). The slow migrating FLAG-CBN band disappeared in the presence of kinase-dead NEK2, suggesting that it is a phosphorylated form of FLAG-CBN (Figure 22A). The slow migrating band of FLAG-CBN$^{4A\ (T35,S36,41,45A)}$ was significantly reduced whereas those of FLAG-CBN^{S36A} was not (Figure 22A). Furthermore, the slow migrating band of FLAG-CBN$^{4A\ (T3,S4,21,22A)}$, which is the phospho-resistant form against PLK1, was also detected (Figure 22A; Lee et al., 2010). These results suggest that NEK2 phosphorylates four specific residues (T35, S36, S41 and S45) of centrobin. Specific NEK2 phosphorylation of centrobin was also confirmed with the Pro-Q diamond blot staining method which specifically detects all the phosphorylated proteins. The results showed that the phospho-centrobin band was detected only when wild-type centrobin was cotransfected with NEK2 (Figure 22B). These results indicate T35, S36, S41, S45 of centrobin are specific phosphorylation sites of NEK2.

The phospho-resistant centrobin against NEK2 stabilizes

microtubules

I generated inducible cell lines in which ectopic centrobin proteins are stably expressed. The immunoblot analysis revealed that FLAG-CBN is by five-fold more abundant than endogenous centrobin even without an induction (Figure 23A). Furthermore, doxycycline induced the FLAG-CBN levels by twenty-fold more (Figure 23A). I prepared several mutant forms of centrobin in which the candidate phosphorylation sites (T35, S36, S41 and S45) are substituted with alanines or glutamates (Figure 23B). Expression of the ectopic FLAG-CBN proteins was induced by doxycycline in the tet-on HeLa cells (Figure 23B).

I performed the nocodazole-resistant assay to examine the effect of centrobin phosphorylation on cytoplasmic microtubule stability of the stable cell lines. The results showed that the FLAG-CBN^{4A}-expressing cells include much more stable microtubules than the control or FLAG-CBN-expressing cells (Figure 24A). The quantitative analysis showed that the nocodazole-resistance of the FLAG-CBN^{4A}–expressing cells increased by two-fold in comparison to the control cells (Figure 24B). The nocodazole-resistance of the FLAG-CBN^{S36A}–expressing cells also increased nearly 40%. However, there is no significant difference in the nocodazole-resistance between the FLAG-CBN-expressing cells and control cells (Figure 24B). The phospho-mimetic mutants (FLAG-CBN^{S36E}, FLAG-CBN^{4E}) also revealed no significant effect on the nocodazole-resistance (Figure 24B). I observed comparable amounts of nocodazole-resistance between two different stable lines of all constructs (Figure 24C). These results revealed that four phosphorylation sites including serine 36 of centrobin are important for the regulation of cytoplasmic microtubule stability

by NEK2.

The nocodazole-resistant activity of FLAG-CBN- and FLAG-CBN^{4A}-expressing cells did not change irrespective of the doxycycline treatment (Figure 24D). However, the nocodazole-resistant activity increased when NEK2 was depleted in the stable cell lines expressing FLAG-CBN and FLAG-CBN^{4A} (Figure 24E). Altogether, these results indicate that NEK2 negatively regulates cytoplasmic microtubule stability through centrobin phosphorylation.

I determined the cell area in the FLAG-CBN-expressing stable cell lines. The results showed that the FLAG-CBN^{4A}-expressing cells spread more than the control and FLAG-CBN-expressing cells (Figure 25A). I quantified the areas of the FLAG-CBN-expressing cells. The results showed that the average area of the FLAG-CBN^{4A}-expressing cells increased about 25% than the control and FLAG-CBN-expressing cells (Figure 25B). The cell migration activity of the FLAG-CBN^{4A}-expressing cells was also enhanced nearly two-fold (Figure 26). These results indicate the phospho-resistant centrobin mutation promotes cell spreading and migration. However, there was no significant difference in the proliferation activity of the phospho-resistant centrobin-expressing cells, compared to the control cells and the wild-type centrobin-expressing cells (Figure 27).

It is known that, unlike NEK2, PLK1 phosphorylation enhances the microtubule stabilization activity of centrobin (Lee et al., 2010). Therefore, I compared the biological activities of the phospho-resistant centrobin mutant of NEK2 with that of PLK1. As expected, the cells with the phospho-resistant centrobin mutant against NEK2 revealed an increase in nocodazole-resistance as well as cell spreading

and migration (Figure 28). However, the phenotypes of the cells with the phospho-resistant centrobin mutant against PLK1 were comparable but not reduced to those of the control cells (Figure 28). These results suggest that NEK2 phosphorylation specifically regulates the functions of centrobin in interphase cells.

NEK2 phosphorylation of centrobin does not affect centriole duplication

I performed the co-immunoprecipitation assay to determine whether the phospho-resistant centrobin mutant have a binding activity to tubulin or not (Gudi et al., 2011). After overexpression was induced by doxycycline treatment, the tubulin interaction of ectopic centrobin was examined by immunoprecipitation with the α-tubulin antibody. As reported, centrobin was pulled down by the α-tubulin antibody (Figure 29). However, there was no difference in the tubulin interaction activity between FLAG-CBN and FLAG-CBN^{4A} (Figure 29).

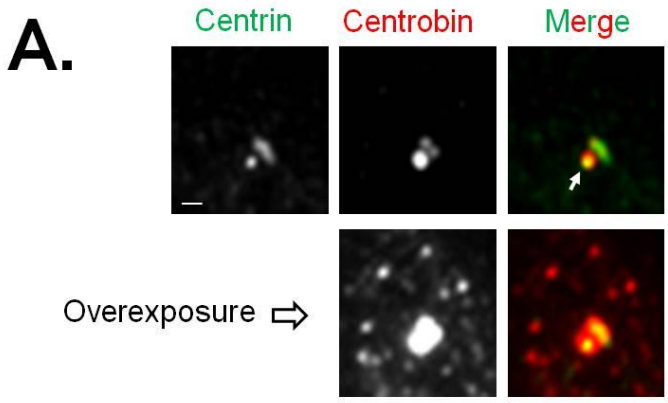
Centrobin was reported to function in centriole duplication (Zou et al., 2005). I tested if FLAG-CBN^{4A} affects centriole duplication or not. Both the wild-type and phospho-resistant centrobin proteins are localized at the centrosomes of interphase and mitotic cells (Figure 30). The centriole number was determined with the cells immunostained with the antibodies for FLAG and CP110 (Figure 31A). The results showed that the centriole number distribution in asynchronous populations of the FLAG-CBN- and FLAG-CBN^{4A}-expressing cells is about the same (Figure 31B). These results indicate that centrobin phosphorylation by NEK2 is critical for microtubule stabilization but not for centriole duplication.

NEK2 phosphorylation of centrobin regulates subcellular distribution of centrobin

I found remarkable difference in the expression pattern of the wild-type and phospho-resistant centrobin in stable cells. FLAG-CBN^{4A} is aggregated, while FLAG-CBN is dispersed around cytoplasm (Figure 32A). I quantified the expression pattern in correlation to the amount of the ectopic proteins after the induction with doxycycline. The expression levels of FLAG-CBN and FLAG-CBN^{4A} are comparable (Figure 32B). Most of FLAG-CBN dispersed around cytoplasm at a low expression level, and form aggregates after the induction (Figure 32C). On the other hand, a significant amount of FLAG-CBN^{4A} already forms aggregates in the beginning, and the amount increases with the doxycycline treatment (Figure 32C). Because the aggregation of ectopic centrobin is thought to reflect the state of centrobin oligomerization, these results suggest NEK2 phosphorylation of centrobin may engage in the centrobin oligomerization.

Figure 7. Subcellular distribution of centrobin.

(A) U2OS cells were coimmunostained with antibodies specific to centrobin (red) and centrin (green). Overexposure of the centrobin signal revealed cytoplasmic centrobin around centrosome (lower panels). Arrow indicates daughter centriole. Scale bar, 1 μm. (B) U2OS cells were transfected with a non-specific control siRNA (*siCTL*) or an siRNA specific to centrobin (*siCBN*). Forty-eight hours later, the cells were fixed and immunostained with antibodies specific to centrobin and γ-tubulin. Scale bar, 20 μm.


A.
Centrin
Centrobin
Merge
Overexposure

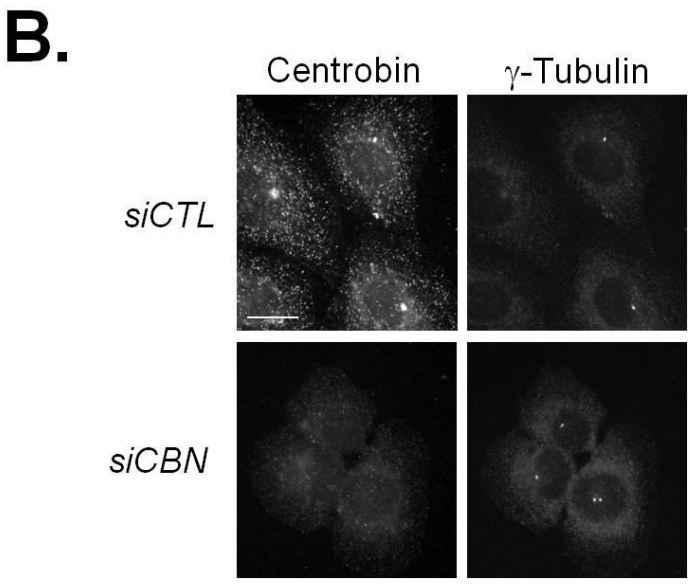

B.
Centrobin
γ-Tubulin
siCTL
siCBN

Figure 8. Cytoplasmic centrobin localizes on the microtubules.

U2OS cells were coimmunostained with antibodies specific to centrobin (red) and α-tubulin (green). Centrobin signals near centrosome (a) and at cell periphery (b) were shown at higher magnification on the right. Scale bars, 10 and 2.5 μm.

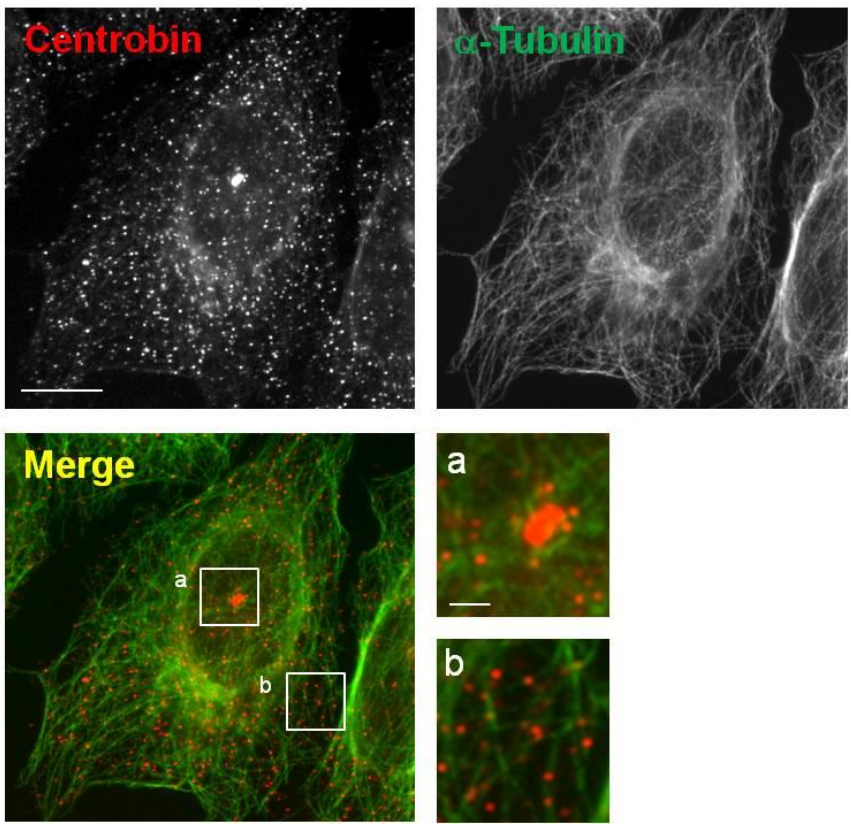

Centrobin
α-Tubulin
Merge
a
b
a
b

Figure 9. Centrobin is associated with acetylated microtubules.

(A) U2OS cells were coimmunostained with antibodies specific to centrobin (red) and acetylated α-tubulin (green). Note that the sizes of centrobin signals are different to each other. Scale bar, 10 μm. (B) U2OS cells were transfected with *pMyc-centrobin*. Twenty-four hours later, the cells were coimmunostained with antibodies specific to the myc tag (red) and acetylated α-tubulin (green). Scale bar, 10 μm.

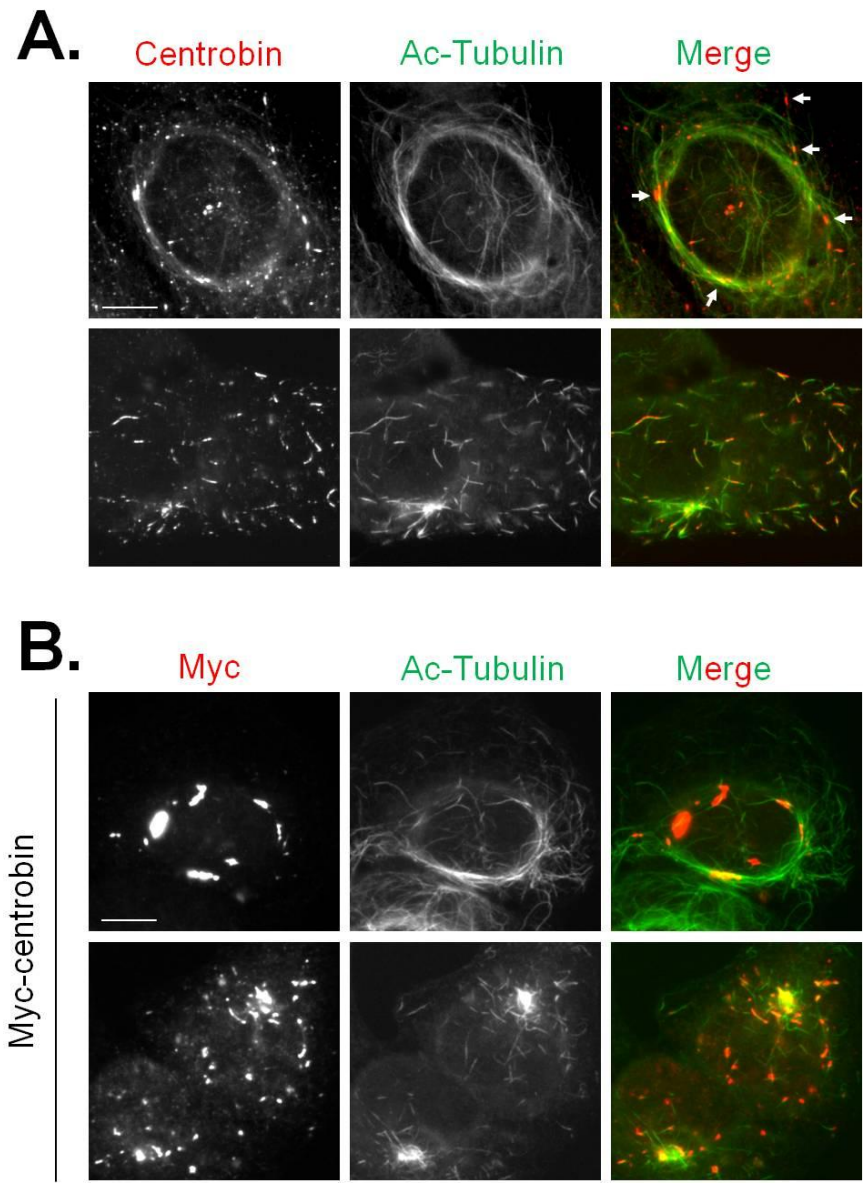

A.
Centrobin
Ac-Tubulin
Merge
B.
Myc
Ac-Tubulin
Merge
Myc-centrobin

Figure 10. Knockdown of NEK2 and centrobin in HeLa, U2OS and RPE1 cells.

HeLa, U2OS and RPE1 cells were transfected with siRNAs specific to NEK2 (*siNEK2*) and centrobin (*siCBN*), along with the control siRNA (*siCTL*). Forty-eight hours later, the cells were subjected to immunoblot analysis with antibodies specific to centrobin, NEK2 and γ-tubulin.

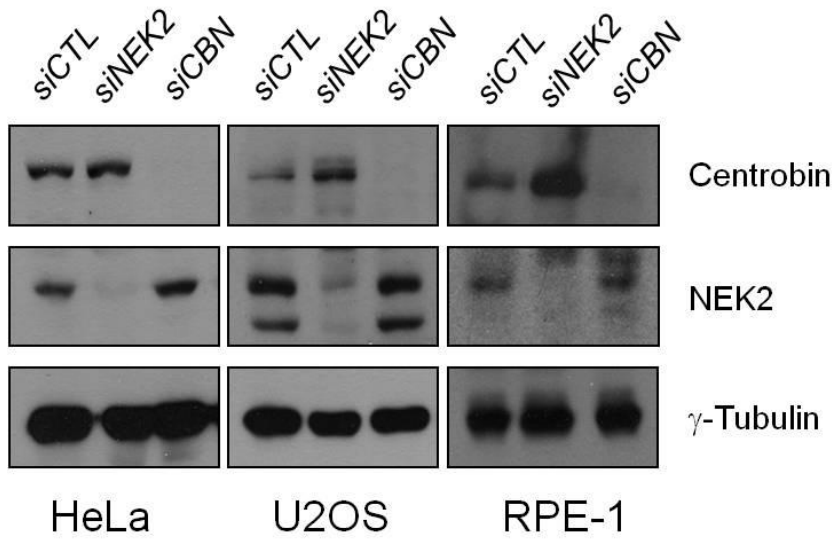

siCTL
siNEK2
siCBN
siCTL
siNEK2
siCBN
siCTL
siNEK2
siCBN
Centrobin
NEK2
γ-Tubulin
HeLa
U2OS
RPE-1

Figure 11. Cell spreading with the NEK2 and centrobin depletion.

(A) Representative phase-contrast images of NEK2- and centrobin-depleted HeLa, U2OS and RPE1 cells. Scale bar, 100 μm. (B) The cells were treated with 2 mM thymidine for 16 h to arrest the cell cycle at S phase and measured the areas of the cells. Over 300 cells per experimental group were counted in 3 independent experiments. The results were presented as means and standard errors. *P<0.001; **P<0.005, in comparison to the control.

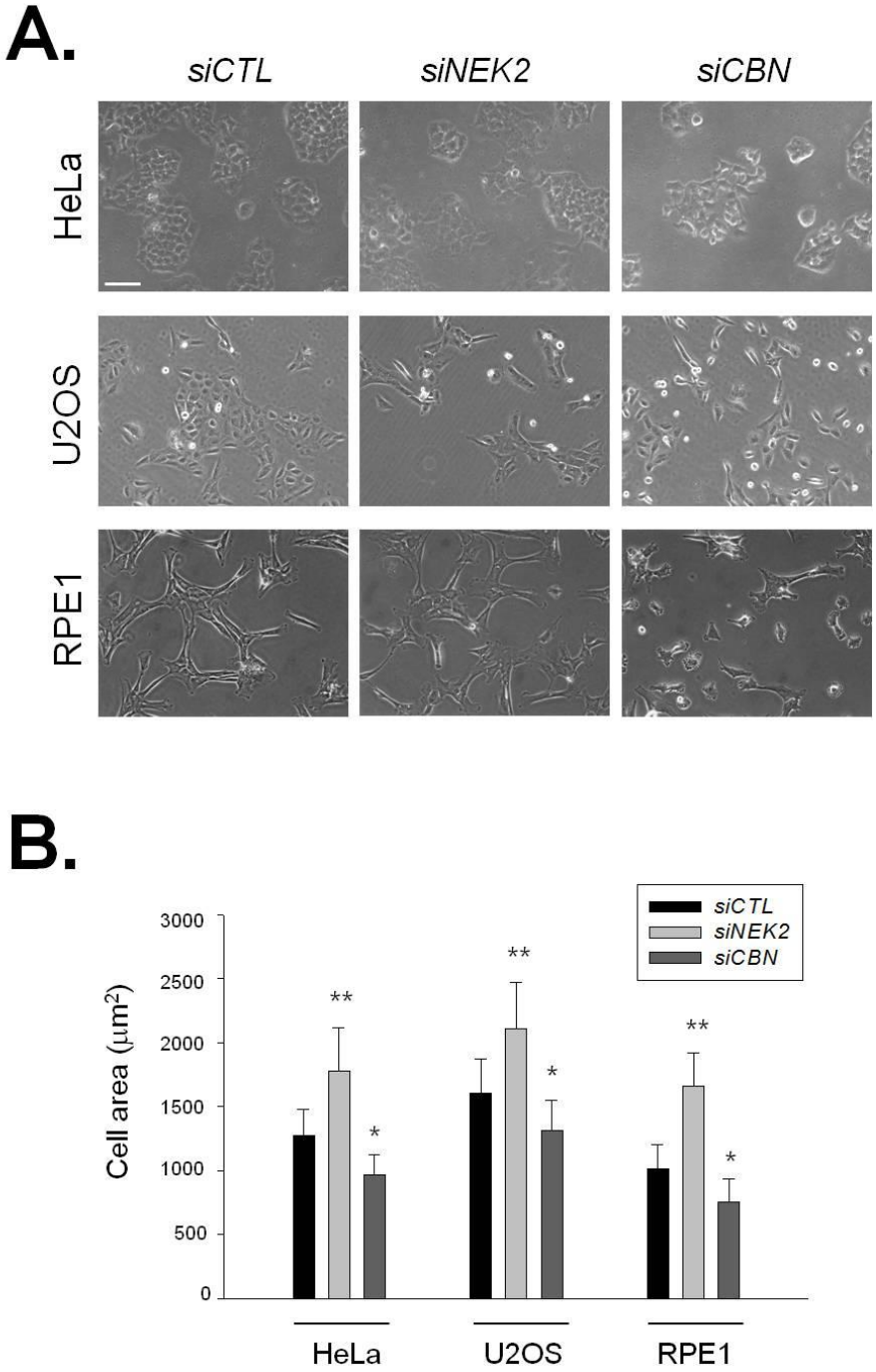
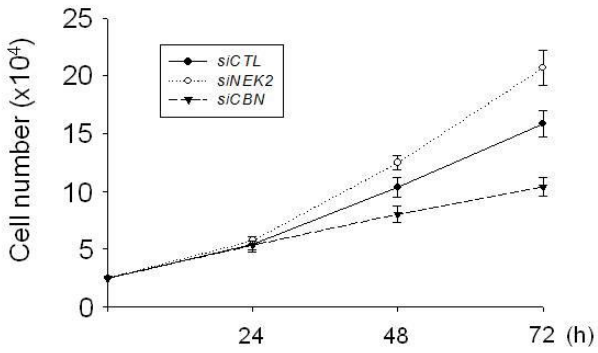
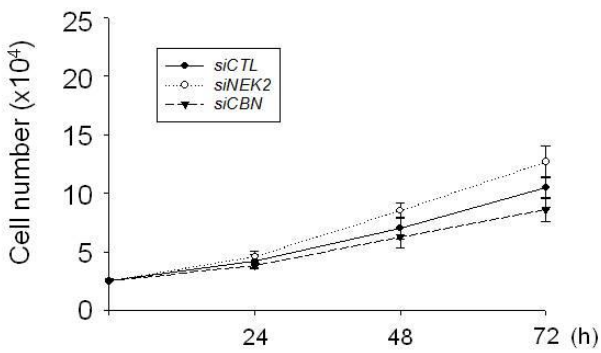

A.
siCTL
siNEK2
siCBN
HeLa
U2OS
RPE1
B.
siCTL
siNEK2
siCBN
Cell area (μm²)
3000
2500
2000
1500
1000
500
0
**
*
**
*
**
*
HeLa
U2OS
RPE1

Figure 12. Proliferation of the NEK2- and centrobin-depleted cells.

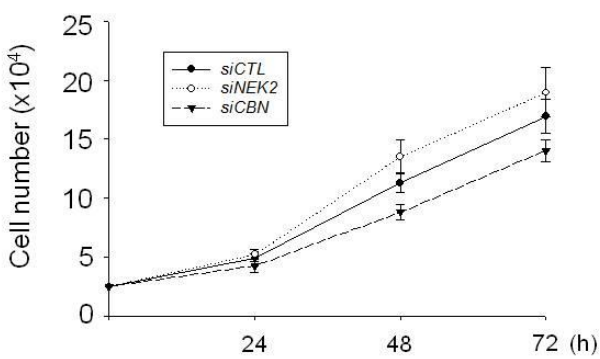
HeLa, U2OS and RPE1 cells were transfected with *siCTL*, *siNEK2* and *siCBN*. Twenty-four hours later, 2.5×10^4 cells were plated on a 4-well dish and cultured for 72 more hours. The number of cells was counted at every 24 h. The results were from two independent experiments and presented as means and standard errors.



<HeLa>



<U2OS>



<RPE1>

Figure 13. Microtubule networks in NEK2- and centrobin-depleted cells.

NEK2- and centrobin-depleted HeLa, U2OS and RPE1 cells were subjected to immunostaining with an antibody specific to α-tubulin. Scale bar, 20 μm.

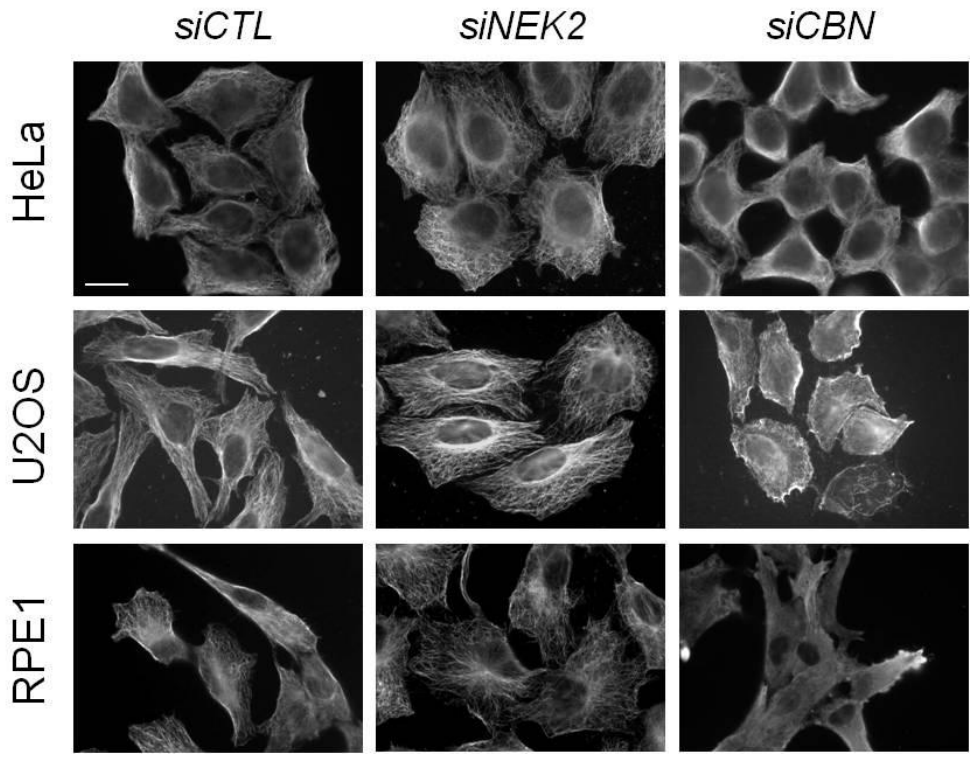

siCTL
siNEK2
siCBN
HeLa
U2OS
RPE1

Figure 14. Immunostaining of centrobin in NEK2- and centrobin-depleted U2OS cells.

NEK2- and centrobin-depleted U2OS cells were subjected to immunostaining with antibodies specific to centrobin (red) and α-tubulin (green). Scale bar, 20 μm.

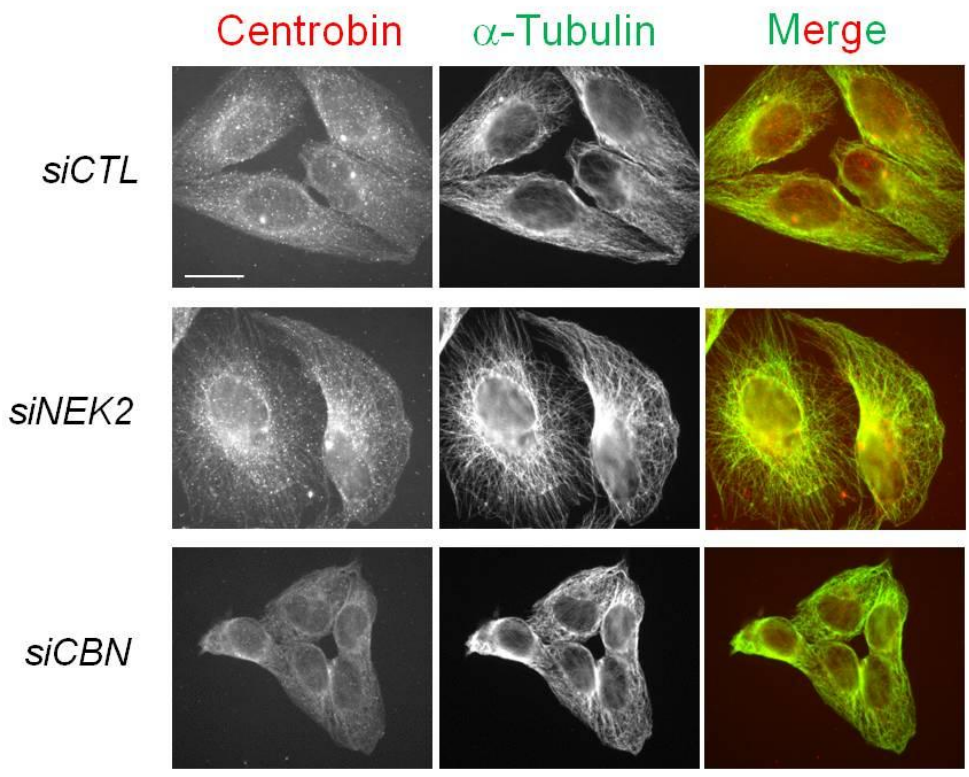

Centrobin
α-Tubulin
Merge
siCTL
siNEK2
siCBN

Figure 15. Actin filament networks in NEK2- and centrobin-depleted cells.

NEK2- and centrobin-depleted HeLa, U2OS and RPE1 cells were subjected to staining with TRITC-conjugated phalloidin. Scale bar, 20 μm.

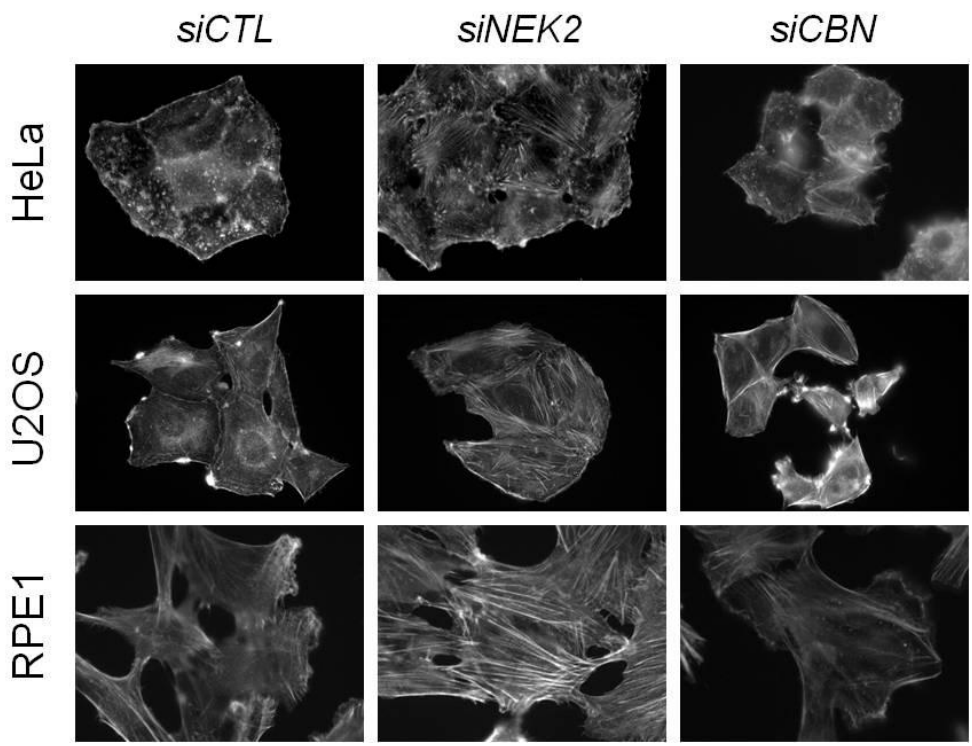

siCTL
siNEK2
siCBN
HeLa
U2OS
RPE1

Figure 16. Cell migration in NEK2- and centrobin-depleted cells.

(A) The NEK2- and centrobin-depleted HeLa cells were cultured for 48 h to reach a confluence. Mitomycin C (10 μg/ml) was treated for 3 h to stop the cell proliferation and wound was made using a yellow pipette tip. The cells were cultured for 24 h further and determined the wound recovery with a phase-contrast microscope. Scale bar, 200 μm. (B) Quantitative analysis after wound recovery assay was performed with the NEK2- and centrobin-depleted HeLa, U2OS and RPE1 cells. The migration distance was measured using the Image-Pro software. The experiments were repeated 3 times and the results were presented as means and standard errors. *$P<0.01$; **$P<0.05$, in comparison to the control.

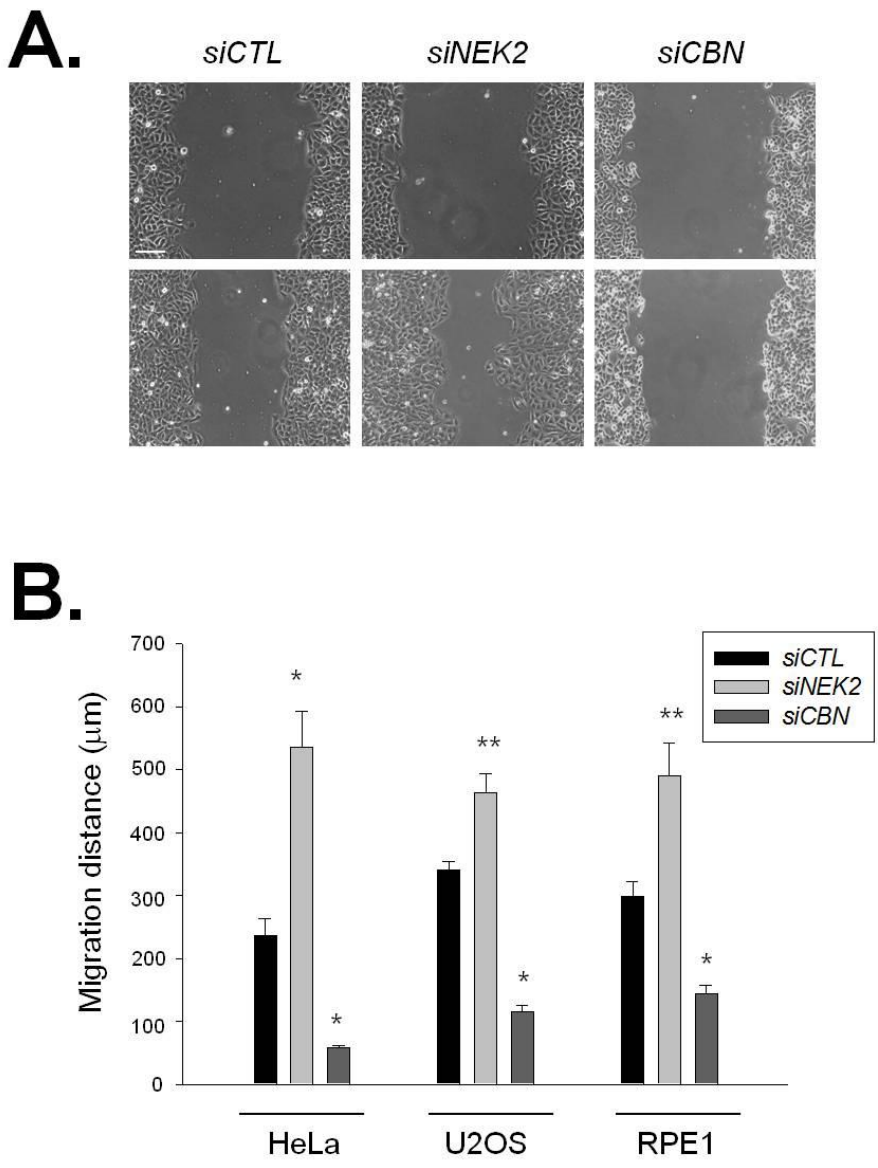
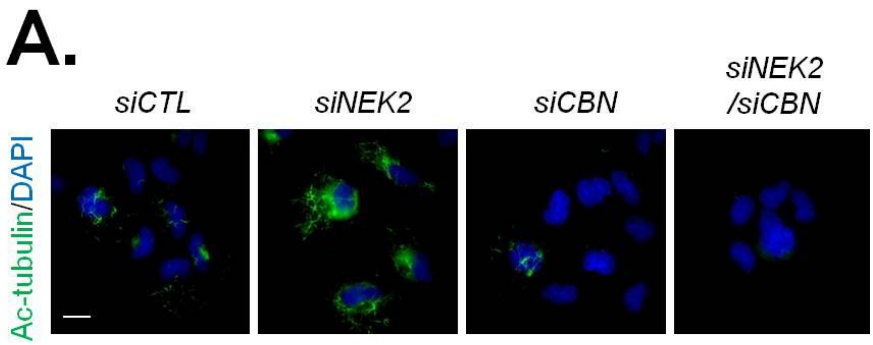
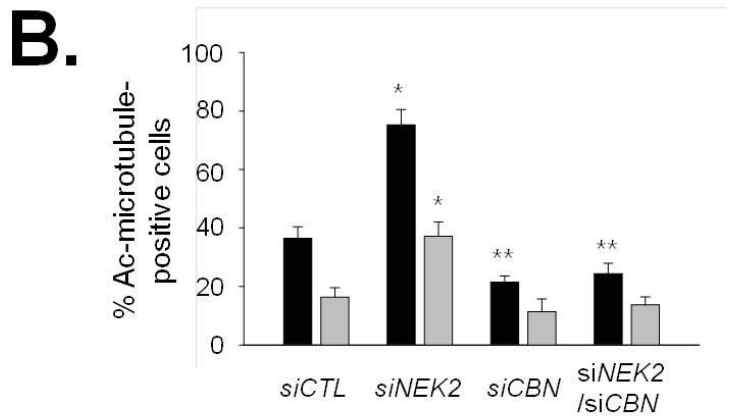

A.
siCTL
siNEK2
siCBN
B.
Migration distance (µm)
700
600
500
400
300
200
100
0
*
**
*
**
*
*
siCTL
siNEK2
siCBN
HeLa
U2OS
RPE1

Figure 17. Microtubule stability in NEK2- and centrobin-depleted cells.

(A) The NEK2- and centrobin-depleted HeLa cells were treated with 2 μM nocodazole for 30 min and immunostained with the acetylated α-tubulin antibody (green). The nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) The cells were treated with 2 μM nocodazole for 30 min (black bar) or 60 min (gray bar). The number of cells with the nocodazole-resistant acetylated tubulin was counted and statistically analyzed. Over 300 cells per experimental group were counted in 3 independent experiments. The results were presented as means and standard errors. *$P<0.005$; **$P<0.05$, in comparison to the control. (C) Intensities of immunostaining signals of the nocodazole-resistant acetylated microtubules were measured densitometrically. The experiments were repeated three times, and 20 cells were analyzed per each experimental group. The immunostaining intensity was determined in an arbitrary unit and the mean values are indicated.


A.
siCTL
siNEK2
siCBN
siNEK2
/siCBN
Ac-tubulin/DAPI


B.
% Ac-microtubule-positive cells
100
80
60
40
20
0
*
*
**
**
siCTL
siNEK2
siCBN
siNEK2
/siCBN

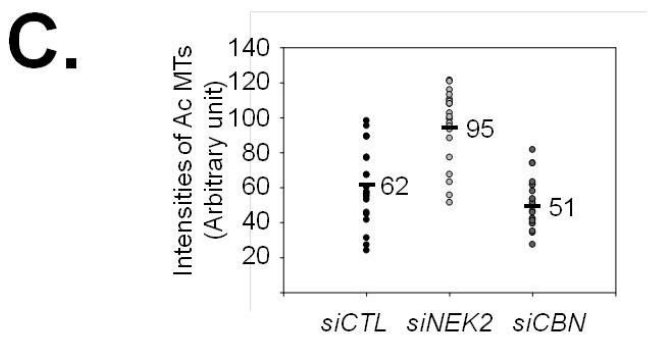
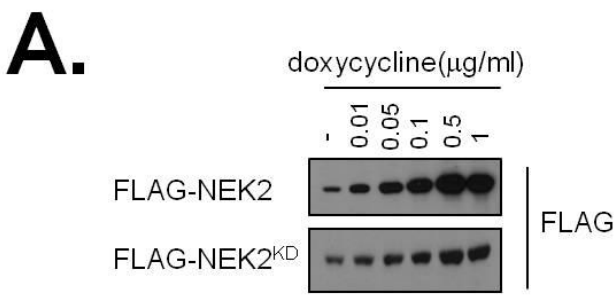

C.
Intensities of Ac MTs
(Arbitrary unit)
140
120
100
80
60
40
20
62
95
51
siCTL
siNEK2
siCBN

Figure 18. The kinase activity of NEK2 is critical for the microtubule stabilizing activity.

(A) We established tet-on HeLa cell lines in which the wild-type and kinase dead (KD) FLAG-NEK2 are stably expressed. Immunoblot analysis was performed to determine FLAG-NEK2 expression with increasing concentrations of doxycycline. (B) The control (*siCTL*) or NEK2-specific (*siNEK2*) siRNA was transfected into the tet-on HeLa cells in which wild-type (WT) or kinase-dead (KD) FLAG-NEK2 are stably expressed. Doxycycline was treated to induce the ectopic NEK2 expression. Immunoblot analysis was performed to determine the endogenous (NEK2 A, B; arrowheads) and ectopic (asterisk) NEK2 levels. The same set of the cells were subjected to the nocodazole-resistant assays. The cells were treated with 2 μM nocodazole for 30 min. The results were presented as means and standard errors. *P<0.005.


A.
doxycycline(μg/ml)
-
0.01
0.05
0.1
0.5
1
FLAG-NEK2
FLAG-NEK2KD
FLAG

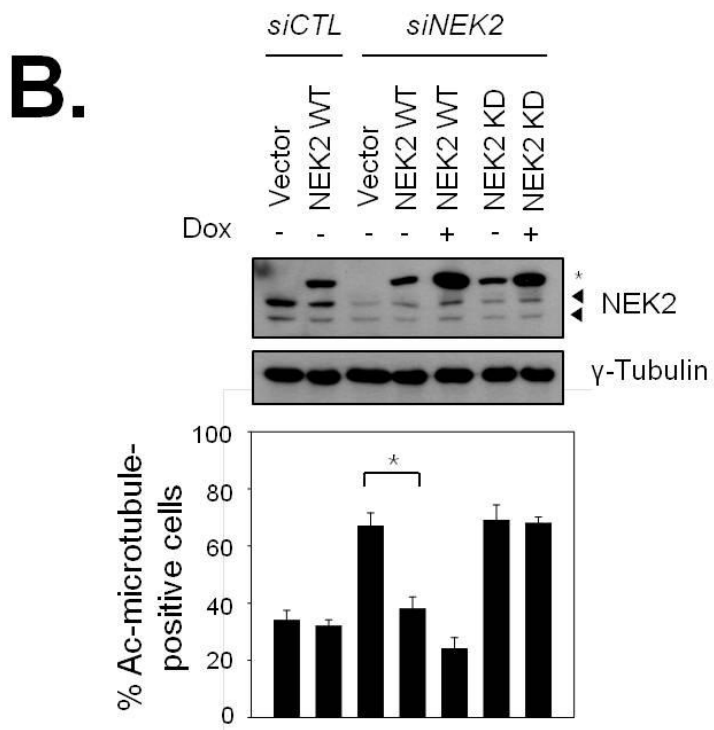

B.
siCTL
siNEK2
Vector
NEK2 WT
Vector
NEK2 WT
NEK2 WT
NEK2 KD
NEK2 KD
Dox
- - - - + - +
NEK2
γ-Tubulin
% Ac-microtubule-positive cells
100
80
60
40
20
0
*

Figure 19. Ectopically expressed centrobin can rescue the microtubule stabilizing activity.

(A) The endogenous centrobin levels were selectively depleted with *siCBN* in the siRNA-resistant centrobin-expressing stable cell line. Forty-eight hours after transfection, the cells were subjected to immunoblot analysis to determine the cellular centrobin levels. The nocodazole-resistant assay was performed with the same set of cells. The cells were treated with 2 μM nocodazole for 30 min. The results were presented as means and standard errors. *$P<0.05$.

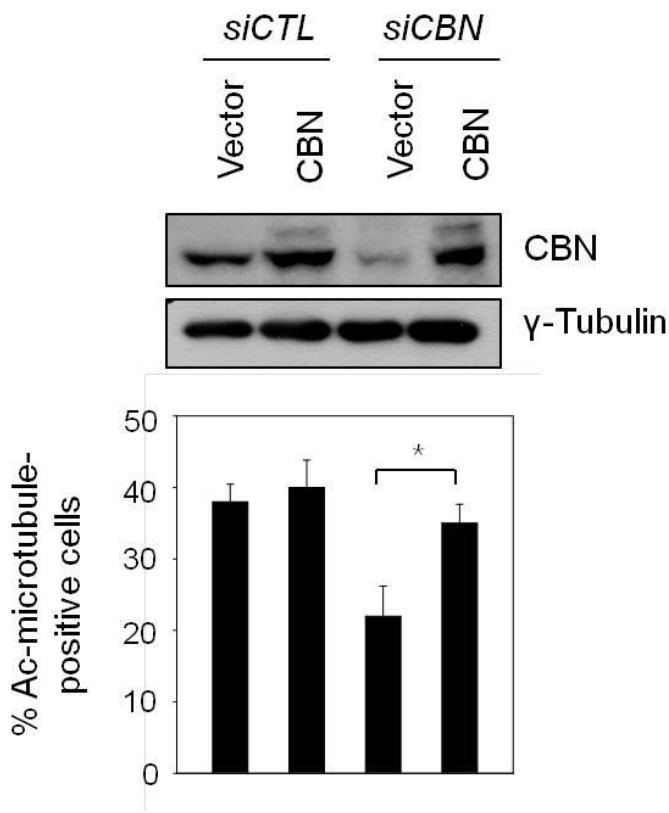
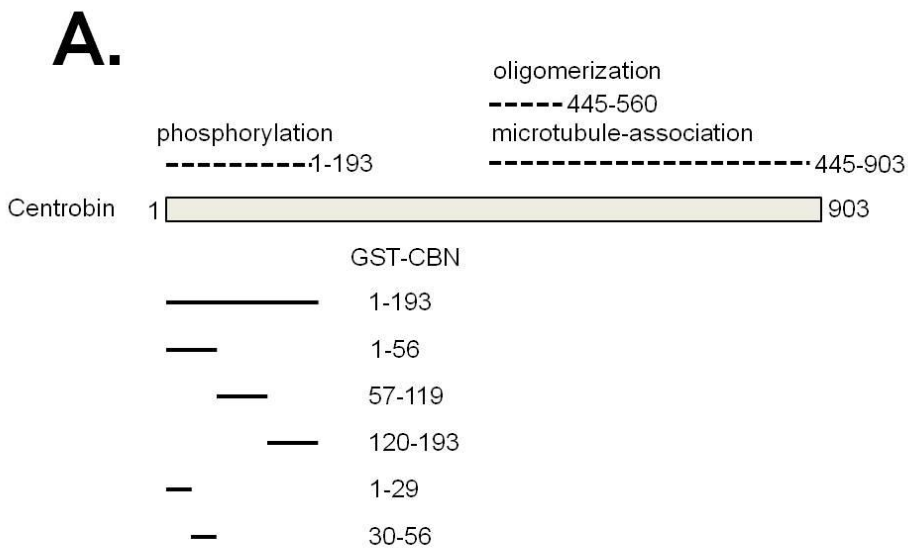

siCTL
siCBN
Vector
CBN
Vector
CBN
CBN
γ-Tubulin
% Ac-microtubule-positive cells
50
40
30
20
10
0
*

Figure 20. In vitro kinase assay of NEK2.

(A) Schematic diagram of the centrobin protein. The 1-193 residues are specifically phosphorylated by NEK2 in vitro. The 445-560 and 445-903 residues are responsible for oligomerization and microtubule binding, respectively (Jeong et al., 2007). (B) In vitro kinase assay was performed with wild-type (WT) or kinase-dead (KD) form of GFP-NEK2 which was expressed and immunoprecipitated from 293T cells. As substrates, truncated mutants of GST-centrobin (GST-CBN) were purified from the bacterial lysates. The amount of the GST-CBN substrates was determined by Coomassie Blue staining. Arrowheads indicate the phosphorylated GST-centrobin proteins.


A.
oligomerization
445-560
phosphorylation
microtubule-association
1-193
445-903
Centrobin
1
903
GST-CBN
1-193
1-56
57-119
120-193
1-29
30-56

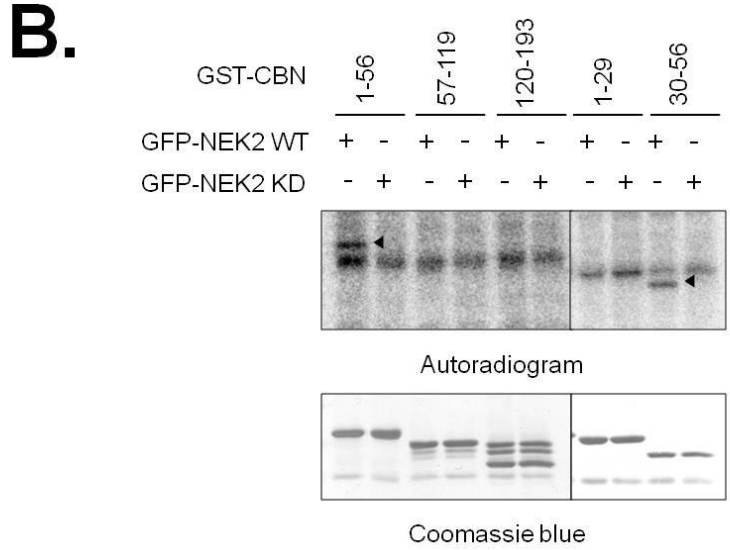
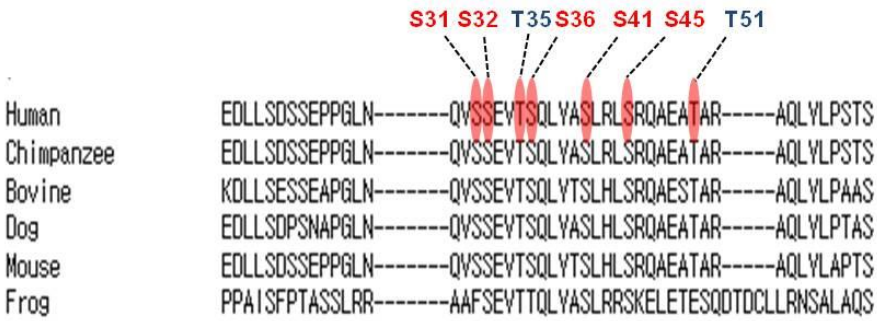

B.
GST-CBN
1-56
57-119
120-193
1-29
30-56
GFP-NEK2 WT + - + - + - + - + -
GFP-NEK2 KD - + - + - + - + - +
Autoradiogram
Coomassie blue

Figure 21. Four sites (T35, S36, S41 and S45) of centrobin are important for phosphorylation by NEK2 in vitro.

(A) There are seven serine/threonine sites within 30-56 residues of centrobin. They are all conserved among mammalian species. (B) In vitro kinase assay of NEK2 was performed with the phospho-resistant mutants of GST-CBN$^{30-56}$ in which each of the serine and threonine residues were substituted with alanines. The amount of the GST-CBN substrates was determined by Coomassie Blue staining. The arrowhead indicates the phosphorylated GST-centrobin proteins.

A.


S31 S32 T35 S36 S41 S45 T51
Human EDLLSDSSEPPGLN-------QVSSEVTSQLYASLRLSRQAEATAR-----AQLYLPSTS
Chimpanzee EDLLSDSSEPPGLN-------QVSSEVTSQLYASLRLSRQAEATAR-----AQLYLPSTS
Bovine KDLLSESSEAPGLN-------QVSSEVTSQLYTSLHLSRQAESTAR-----AQLYLPAAS
Dog EDLLSDPSNAPGLN-------QVSSEVTSQLYASLHLSRQAEATAR-----AQLYLPTAS
Mouse EDLLSDSSEPPGLN-------QVSSEVTSQLYTSLHLSRQAEATAR-----AQLYLAPTS
Frog PPAISFPTASSLRR-------AAFSEVTTQLYASLRRSKELETESQDTDCLLRNSALAQS

B.

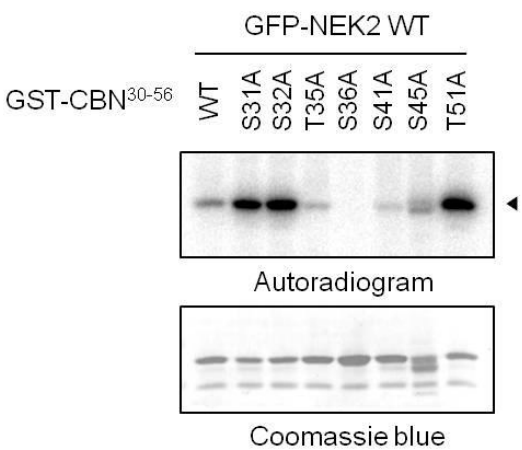
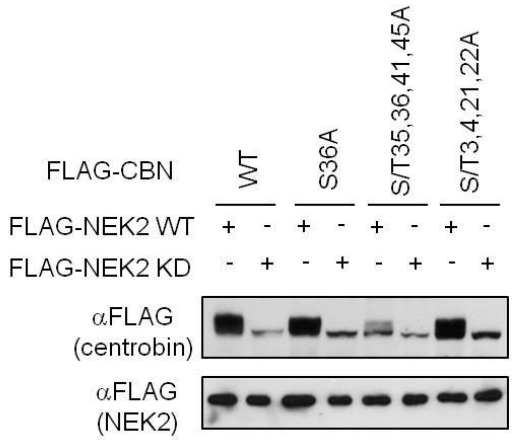

GFP-NEK2 WT
GST-CBN30-56
WT
S31A
S32A
T35A
S36A
S41A
S45A
T51A
Autoradiogram
Coomassie blue

Figure 22. In vivo NEK2 phosphorylation of centrobin.

(A) Wild-type or phospho-resistant mutants of FLAG-CBN were co-transfected into 293T cells along with wild-type or kinase-dead FLAG-NEK2. The alanine-substituted point mutants of centrobin include the sites for NEK2 (S36A, and S/T35,36,41,45A) and for PLK1 (S/T3,4,21,22A). The cell lysates were subjected to immunoblot analysis with the FLAG antibody for detection of both centrobin and NEK2 proteins. (B) The wild-type or phospho-resistant mutant (S/T35,36,41,45A) of FLAG-CBN were co-transfected into 293T cells with the wild-type or kinase-dead FLAG-NEK2, purified, and subjected to Pro-Q Diamond blot staining. The amount of the purified FLAG-CBN was determined by Coomassie blue staining. The arrowhead indicates FLAG-CBN.

A.


FLAG-CBN
WT
S36A
S/T35,36,41,45A
S/T3,4,21,22A
FLAG-NEK2 WT + - + - + - + -
FLAG-NEK2 KD - + - + - + - +
αFLAG (centrobin)
αFLAG (NEK2)

B.

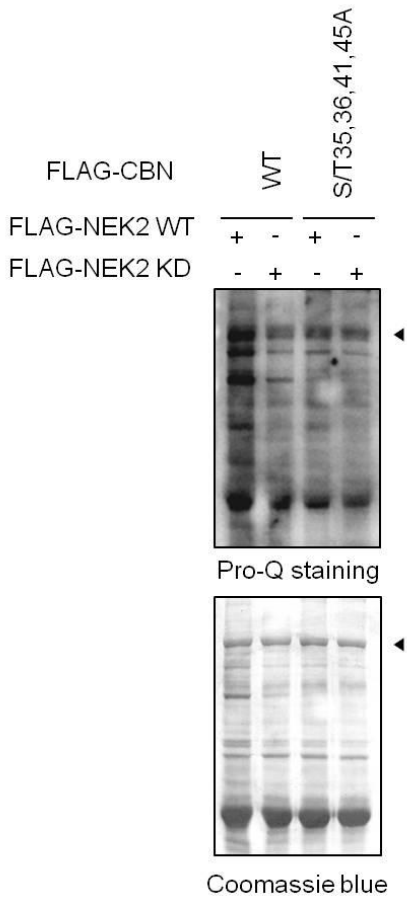
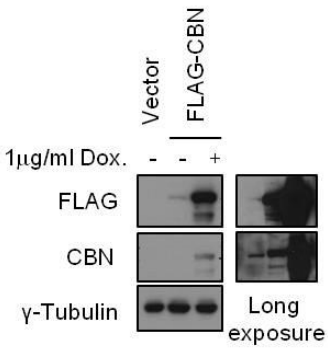

FLAG-CBN
WT
S/T35,36,41,45A
FLAG-NEK2 WT + - + -
FLAG-NEK2 KD - + - +
Pro-Q staining
Coomassie blue

Figure 23. Generation of the tet-on stable HeLa cell lines expressing FLAG-tagged centrobin.

(A) Overexpression of FLAG-CBN was induced by 1 μg/ml doxycycline. Twenty-four hours after induction, the cells were subjected to immunoblot analysis with the antibodies specific to centrobin, FLAG and γ-tubulin. The same blots were overexposed to compare the levels of endogenous centrobin with ectopic FLAG-CBN. (B) We established tet-on HeLa cells which stably express FLAG-CBN with point mutations [S36A, 4A (S/T35,36,41,45A), S36E, 4E (S/T35, 36,41,45E)]. Immunoblot analysis was performed to determine FLAG-CBN expression with increasing concentrations of doxycycline for 24 h.

A.


Vector
FLAG-CBN
1μg/ml Dox. - - +
FLAG
CBN
γ-Tubulin
Long exposure

B.

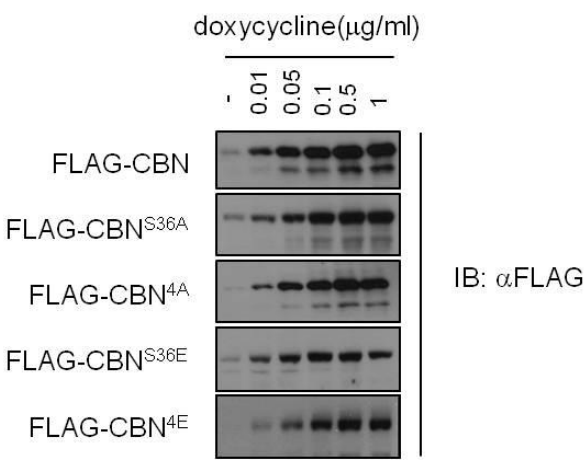
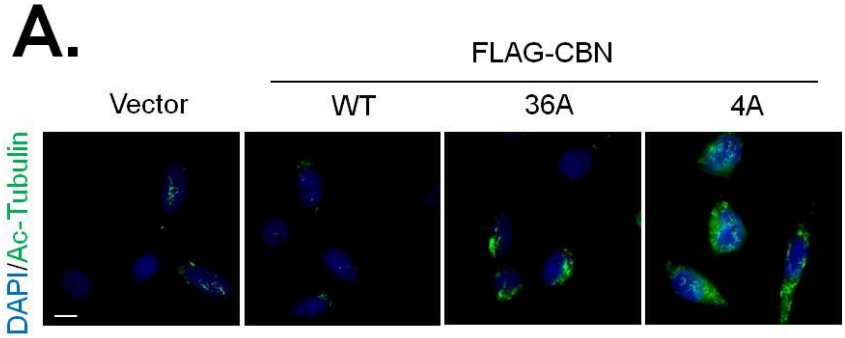
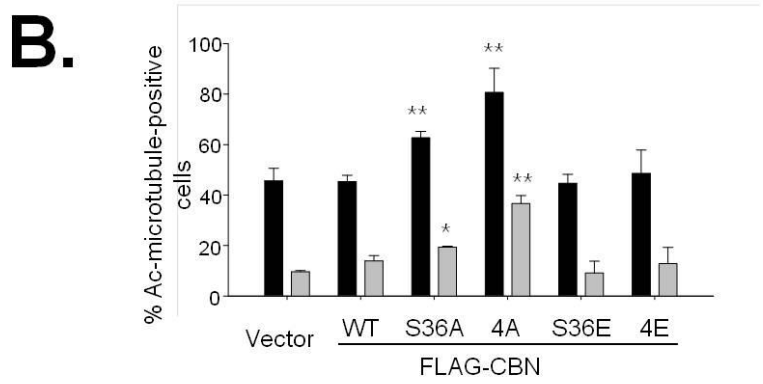
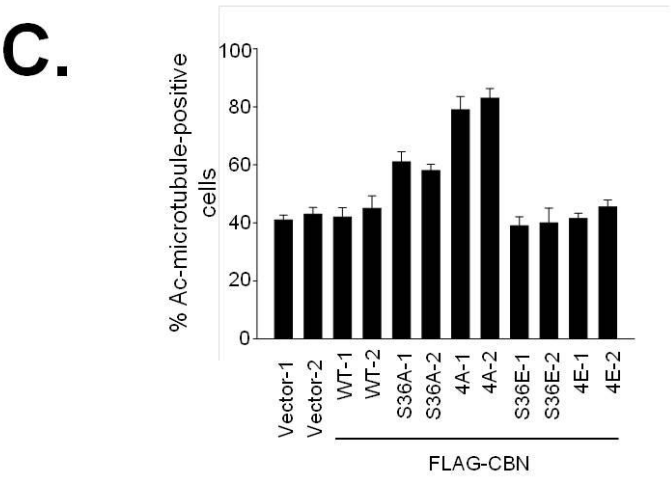

doxycycline(μg/ml)
-
0.01
0.05
0.1
0.5
1
FLAG-CBN
FLAG-CBN^{S36A}
FLAG-CBN^{4A}
FLAG-CBN^{S36E}
FLAG-CBN^{4E}
IB: αFLAG

Figure 24. The microtubules become stabilized in cells expressing the phospho-resistant centrobin mutants.

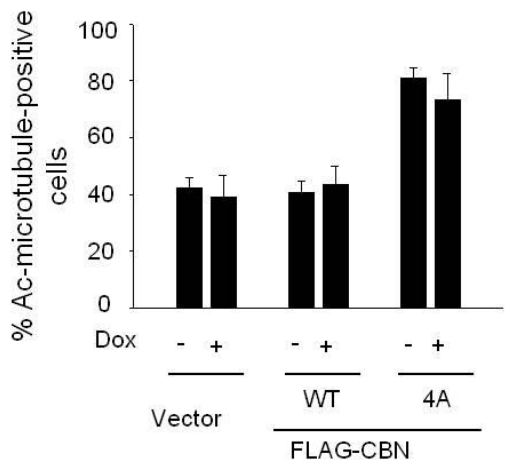
(A) The cells were immunostained with the acetylated α-tubulin antibody (green) after treatment of 2 μM nocodazole for 30 min. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (B) The FLAG-CBN-expressing cells were treated with nocodazole for 30 min (black bar) or 60 min (gray bar), and the number of cells with the nocodazole-resistant acetylated tubulin was counted. (C-E) The nocodazole-resistant assay was performed with the 2 μM nocodazole treatment for 30 min. The stable cell lines cell lines includes FLAG-CBN with the point mutations at NEK2 phosphorylation sites (C). The nocodazole-resistant activity of FLAG-CBN^{4A} was determined when its expression level was induced by doxycycline (D). The nocodazole-resistant activities of FLAG-CBN and FLAG-CBN^{4A} were determined in the NEK2-depleted cells (E). The results in this figure were repeated three times and over 300 cells were counted for each experimental group. The results were presented as means and standard errors. *$P<0.05$; **$P<0.01$, in comparison to the control vector.


A.
FLAG-CBN
Vector
WT
36A
4A
DAPI/Ac-Tubulin


B.
% Ac-microtubule-positive cells
100
80
60
40
20
0
**
**
*
**
Vector
WT
S36A
4A
S36E
4E
FLAG-CBN


C.
% Ac-microtubule-positive cells
100
80
60
40
20
0
Vector-1
Vector-2
WT-1
WT-2
S36A-1
S36A-2
4A-1
4A-2
S36E-1
S36E-2
4E-1
4E-2
FLAG-CBN

D.


% Ac-microtubule-positive cells
100
80
60
40
20
0
Dox
- + - + - +
Vector
WT
4A
FLAG-CBN

E.

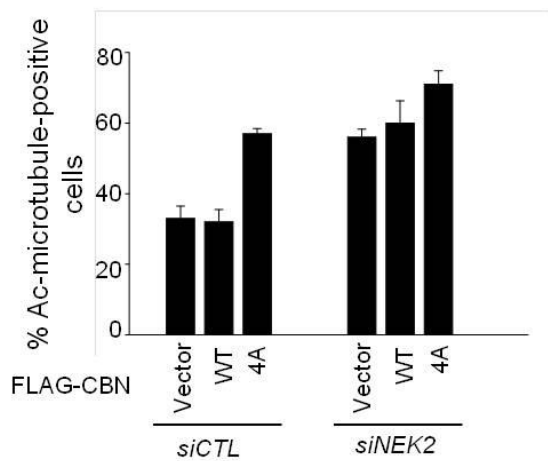
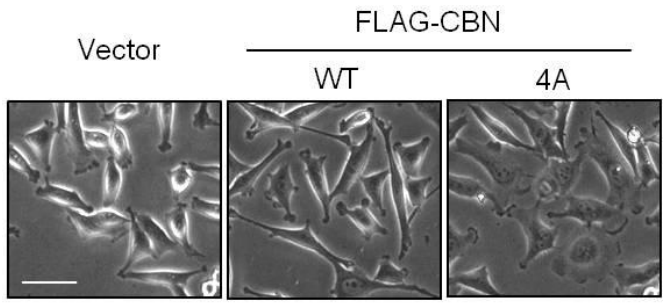

% Ac-microtubule-positive cells
80
60
40
20
0
FLAG-CBN
Vector
WT
4A
Vector
WT
4A
siCTL
siNEK2

Figure 25. An increase in cell spreading with FLAG-CBN^{4A} expression.

(A) The FLAG-CBN- and FLAG-CBN^{4A}–expressing cells were arrested at S phase with treatment of 2 mM thymidine for 16 h. Representative phase-contrast images were taken. Scale bar, 40 μm. (B) The cell area was measured using Image-Pro software. The experiments were repeated three times and over 200 cells were counted per experimental group. The results were presented as means and standard errors. *P<0.05, in comparison to the control vector.

A.


Vector
FLAG-CBN
WT
4A

B.

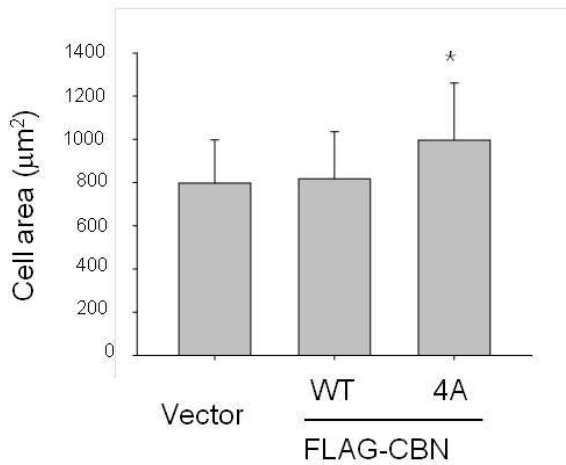
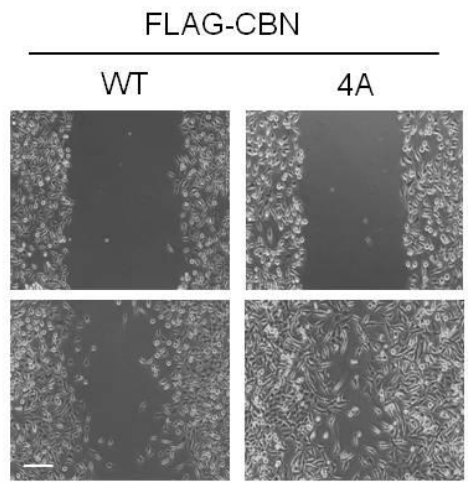

Cell area (μm²)
1400
1200
1000
800
600
400
200
0
*
Vector
WT
4A
FLAG-CBN

Figure 26. The phospho-resistant centrobin mutant increases cell migration.

The FLAG-CBN- and FLAG-CBN^{4A}–expressing cells were cultured to reach a confluence and treated with mitomycin C to block the cell proliferation. Wound was made using a yellow pipette tip. The cells were cultured for 24 h further and the wound recovery rate was determined with a phase-contrast microscope. The results were repeated three times and presented as means and standard errors. *$P<0.01$, in comparison to the control vector.

A.


FLAG-CBN
WT
4A

B.

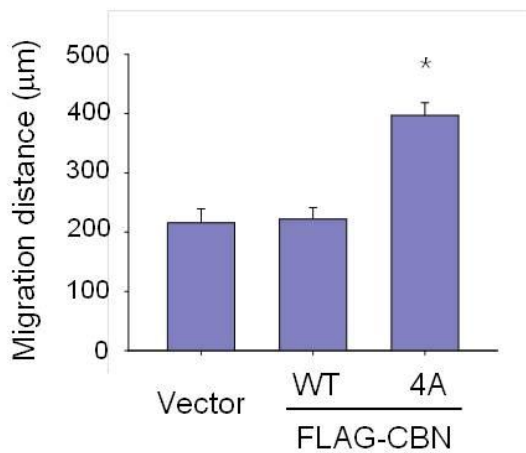

Migration distance (μm)
500
400
300
200
100
0
*
Vector
WT
4A
FLAG-CBN

Figure 27. The stable cell lines with phospho-resistant centrobin mutants proliferate efficiently.

The cell proliferation activities in the FLAG-CBN-expressing cells were determined for up to 72 h. The number of cells was counted at every 24 h. The results were from two independent experiments and presented as means and standard errors.

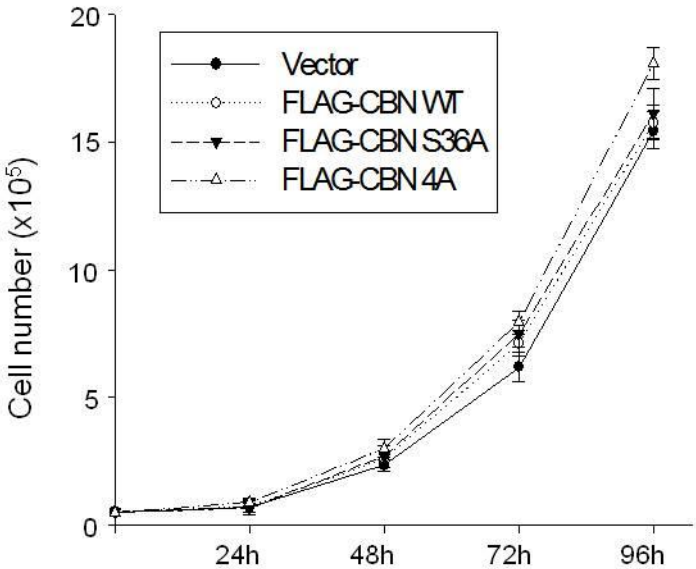
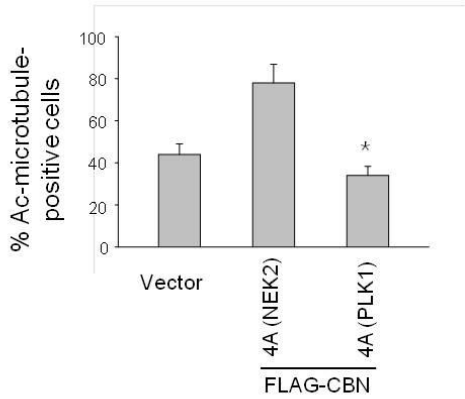

Vector
FLAG-CBN WT
FLAG-CBN S36A
FLAG-CBN 4A
Cell number (x10^5)
20
15
10
5
0
24h
48h
72h
96h

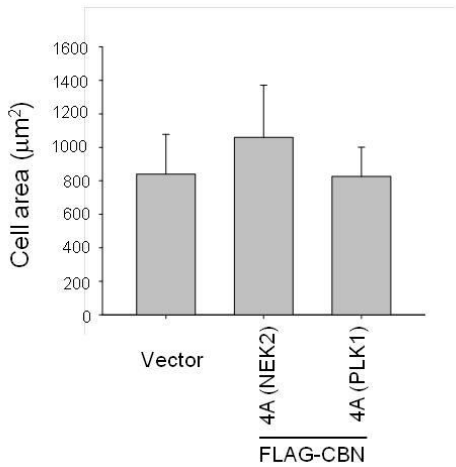
Figure 28. Microtubule stability, cell spreading and migration activity of the cells with the centrobin mutant proteins resistant to NEK2 and PLK1 phosphorylation.

(A) The nocodazole-resistance assay was performed with the cells expressing phospho-resistant FLAG-CBN against NEK2 (S36A, and S/T35,36,41,45A) and against PLK1 (S/T3,4,21,22A). Over 300 cells were counted for each experimental group in two independent experiments. (B) The cells were treated with thymidine for 16 h to arrest the cell cycle at S phase and the cell area was measured using Image-Pro software. Over 200 cells were counted for each experimental group in two independent experiments. (C) The wound recovery assay was performed. The results were obtained from two independent experiments. The results in this figure were presented as means and standard errors. *$P<0.01$, in comparison to the control vector.

A.


% Ac-microtubule-positive cells
100
80
60
40
20
0
*
Vector
4A (NEK2)
4A (PLK1)
FLAG-CBN

B.


Cell area (μm²)
1600
1400
1200
1000
800
600
400
200
0
Vector
4A (NEK2)
4A (PLK1)
FLAG-CBN

C.

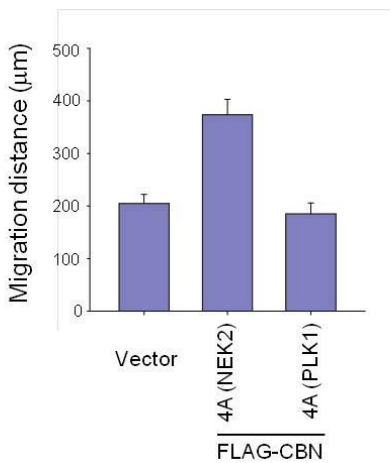

Migration distance (μm)
500
400
300
200
100
0
Vector
4A (NEK2)
4A (PLK1)
FLAG-CBN

Figure 29. Centrobin interaction with tubulin

The α-tubulin was immunoprecipitated in cells expressing FLAG-CBN and FLAG-CBN^{4A} against NEK2. The coimmunoprecipitated FLAG-CBN was detected with the FLAG antibody.

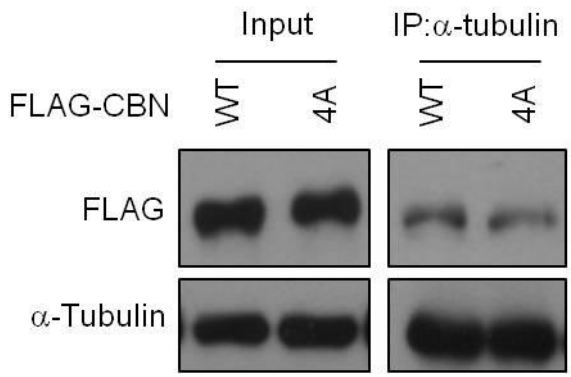

Input
IP:α-tubulin
FLAG-CBN
WT
4A
WT
4A
FLAG
α-Tubulin

Figure 30. Centrosomal localization of the ectopic centrobin

The FLAG-CBN- or FLAG-CBN^{4A}-expressing mitotic and interphase cells were immunostained with antibodies specific to FLAG and γ-tubulin. Scale bar represents 20 μm.

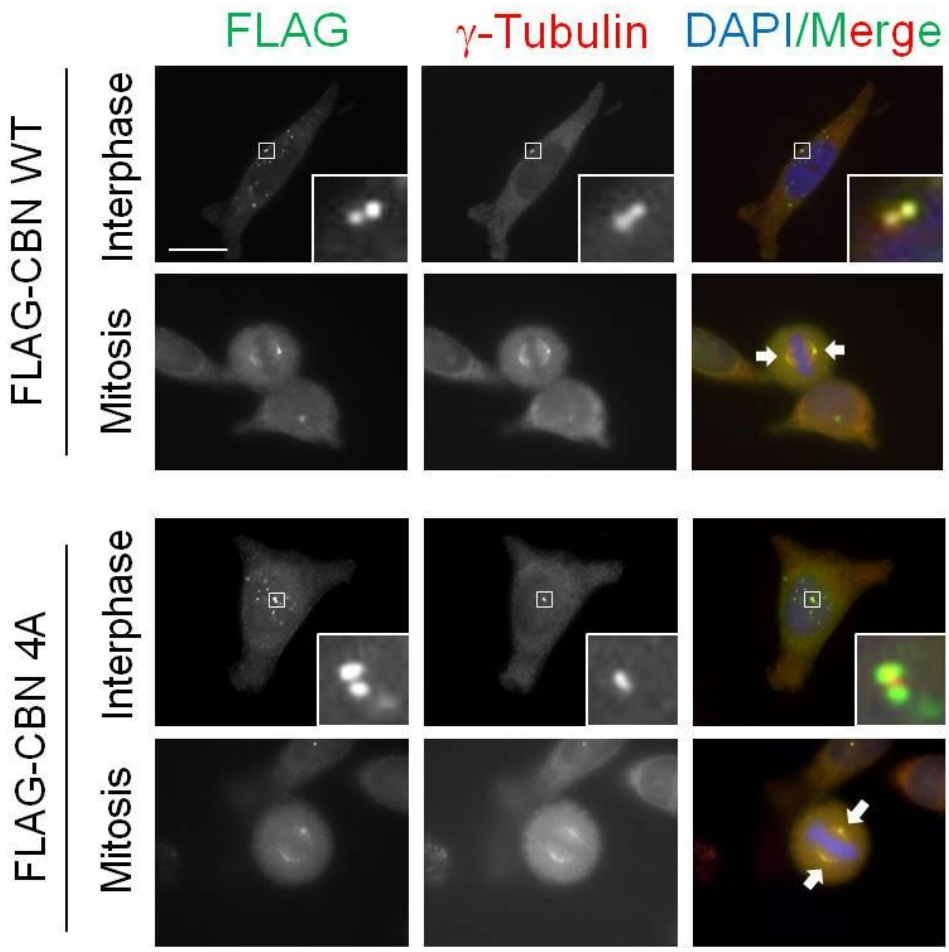
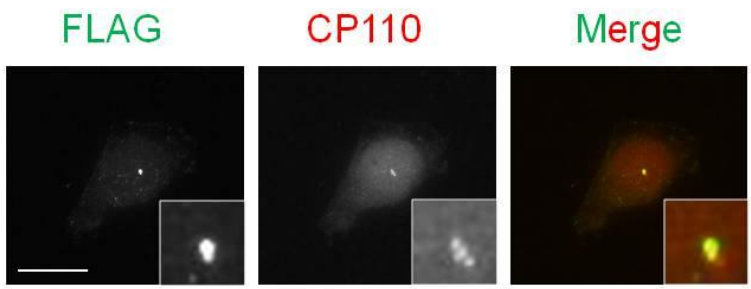

FLAG
γ-Tubulin
DAPI/Merge
FLAG-CBN WT
Interphase
Mitosis
FLAG-CBN 4A
Interphase
Mitosis

Figure 31. Centriole duplication occurs in cells expressing phospho-resistant centrobin.

(A) The FLAG-CBN- and FLAG-CBN^{4A}–expressing cells were immunostained with antibodies specific to FLAG and CP110. Scale bar represents 10 μm. (B) The FLAG-CBN-expressing cells were treated with 2mM thymidine for 16 h to arrest the cell cycle at S phase, and the number of centrioles was counted. The experiments were repeated two times and over 200 cells were counted per experimental group.

A.


FLAG
CP110
Merge

B.

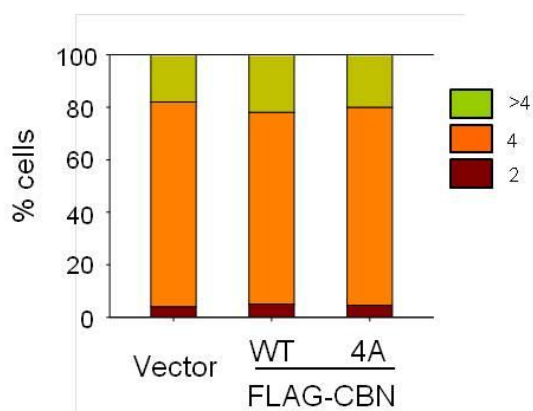
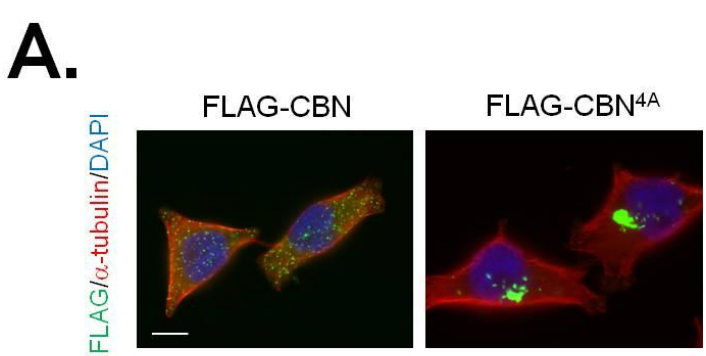
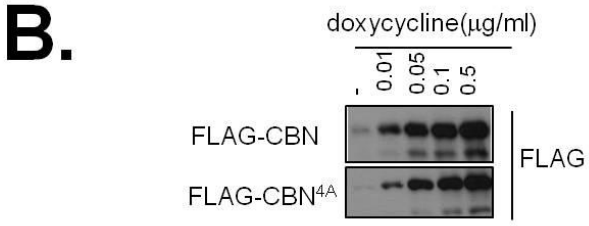

100
80
60
40
20
0
% cells
>4
4
2
Vector
WT
4A
FLAG-CBN

Figure 32. Subcellular distribution of the phospho-resistant FLAG-CBN.

(A) Expression of the stably transfected FLAG-CBN and FLAG-CBN^{4A} was induced with 0.1 μg/ml doxycycline. Twenty-four hours later, the cells were immunostained with antibodies specific to FLAG (green) and α-tubulin (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) The expression levels of FLAG-CBN according to the concentration of doxycycline were determined by immunoblot analysis using FLAG-antibody. (C) Subcellular distribution of the FLAG-CBN and FLAG-CBN^{4A} proteins in doxycycline-treated cells was categorized into three groups; perinuclear aggregation, cytoplasmic dispersion or both. Over 200 cells were counted per experimental group.


A.
FLAG-CBN
FLAG-CBN4A
FLAG/α-tubulin/DAPI


B.
doxycycline(μg/ml)
-
0.01
0.05
0.1
0.5
FLAG-CBN
FLAG-CBN4A
FLAG

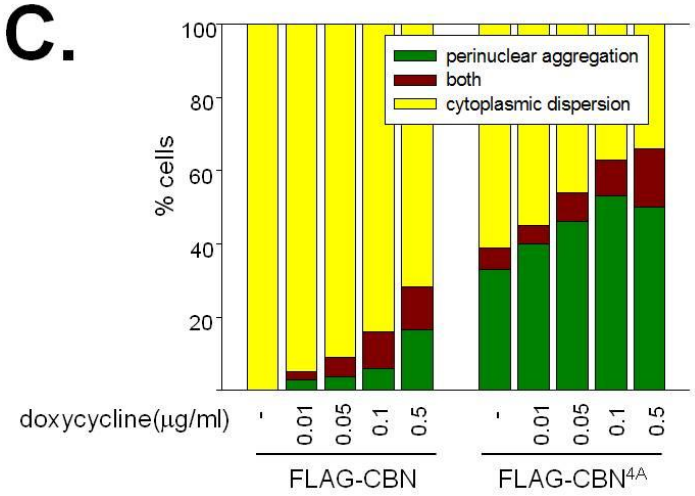
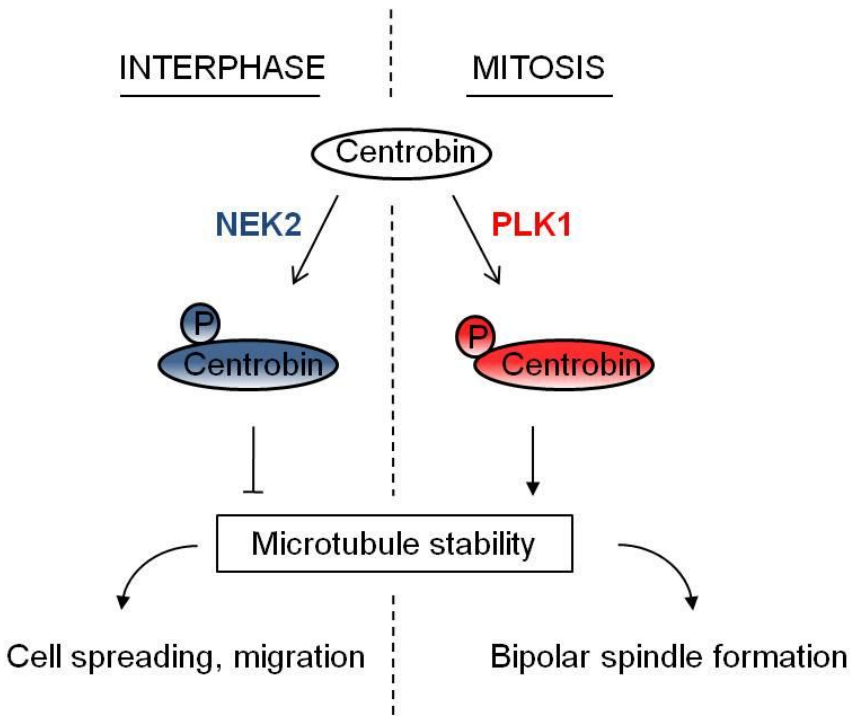

C.
100
80
60
40
20
% cells
perinuclear aggregation
both
cytoplasmic dispersion
doxycycline(μg/ml)
-
0.01
0.05
0.1
0.5
FLAG-CBN
FLAG-CBN4A

Figure 33. Model.

Centrbin is phosphorylated by both NEK2 and PLK1 in a cell cycle stage-specific manner. During interphase, the level of cytoskeletal microtubule stability is determined according to the the level of centrobin phosphorylation by NEK2. This affects cellular activity such as cell spreading and migration. On the other hand, PLK1 phosphorylates and activates centrobin for bipolar spindle formation during mitosis.


INTERPHASE
MITOSIS
Centrobin
NEK2
PLK1
P
Centrobin
P
Centrobin
Microtubule stability
Cell spreading, migration
Bipolar spindle formation

DISCUSSION

In this study, I confirmed the presence of the cytoplasmic centrobin which is hardly detectable in the centrobin-depleted cells. The cytoplasmic centrobin forms granules on the microtubules, and sometimes aggregated along with the stable microtubules which are stained with the antibody specific for acetylated tubulin. This expression pattern is consistent with the proposed function of centrobin for stabilization of microtubules (Jeong et al., 2007; Lee et al., 2010).

A few lines of experimental evidences support the notion that centrobin stabilizes microtubules. First, overexpression of centrobin induces stable microtubule bundles (Jeong et al., 2007). Second, centrobin enhances in vitro microtubule formation but not elongation (Lee et al., 2010). Third, the cellular microtubule network is significantly weakened in the centrobin-depleted cells. As results, the centrobin-depleted cells are shrunk, less motile, and ready to die. Finally, the present work showed that the cytoplasmic centrobin often accumulated and colocalized with stable microtubules at the perinuclear region. The perinuclear localization of centrobin reminds of doublecortin which outlines a cage-like fibrillar bundle encapsulating the nucleus in migrating neurons (Tanaka et al., 2004). Centrobin might have similar functions with doublecortin in nucleation and stabilization of microtubules and cell migration. It is well-known that centrobin is localized at the daughter centriole. Therefore, centrobin might also stabilize nascent procentrioles during centriole biogenesis (Zou et al., 2005; Jeong et al., 2007).

The association patterns of centrobin with stable microtubules seem to be very unique and unprecedented. Although most of cytoplasmic centrobin signals appeared as small granules, they often showed various sizes of elongated, large signals along with stable microtubules. From this observation, I guessed that centrobin moves along the microtubule network repeating accumulation and dispersion, and the microtubules organization and stabilization are affected by these dynamic movements of centrobin. In addition, I observed that centrobin specifically bound to the short fragments of stable microtubules which are dispersed in the cytoplasm or gather at the perinuclear region. So, it will be very interesting to investigate about the reorganization mechanism of stable microtubules related with centrobin.

Centrobin was initially identified as a substrate of NEK2 (Jeong et al., 2007). Here, I revealed that NEK2 phosphorylation controls the microtubule stabilizing activity of centrobin. It is striking that the phospho-resistant mutant of centrobin mimics the knockdown phenotypes of NEK2, which is quite opposite to those of centrobin. These results suggest that NEK2 inhibits the microtubule stabilizing activity of centrobin.

However, I found that overexpression of wild-type centrobin, irrespective of its amount, does not enhance the microtubule stability in interphase cells. Whereas the cellular microtubule stability increased almost two-fold just by the overexpression of phospho-resistant centrobin which is five-fold as many as endogenous centrobin. These results can be explained that a limited population of centrobin is involved in microtubule stabilization and they are regulated by NEK2 enough. Therefore, the important thing for microtubule stabilization may be not the amount of centrobin but

the proportion of unphophorylated centrobin within cells.

It is interesting that centrobin is also phosphorylated by PLK1 (Lee et al., 2010). NEK2 and PLK1 specifically phosphorylate at two distinct sites of centrobin and generate opposite outcomes: NEK2 suppresses microtubule stabilizing activity of centrobin, while PLK1 enhances it (Lee et al., 2010). It remains to be investigated how the centrobin activity is controlled by two protein kinases in opposite manners. It is possible that NEK2 and PLK1 functions in cell cycle stage-specific manners (Figure 33). The microtubule stabilizing activity in interphase cells may be under control of NEK2 for cell morphology and migration. However, in mitotic cells, PLK1 enhances the centrobin activity for stabilization of mitotic spindles. In consistent with this view, the phospho-resistant mutant of centrobin against PLK1 has little effects on microtubule stabilization, cell spreading and motility in interphase cells (Figure 28).

How does NEK2 phosphorylation of centrobin affect microtubule stability? I think that NEK2 might control dynamic movement of centrobin along the microtubule network. In fact, centrobin had a tendency to disperse along the microtubule network, whereas the phospho-resistant centrobin had a tendency to aggregate around nucleus (Figure 32). The results can be explained as two ways. An explanation is that the NEK2 phosphorylation affects the subcellular localization of centrobin, and the perinuclear localization of centrobin might be favorable for stability of the cytoplasmic microtubule network. Doublecortin and Lis1 which are known to be important for neuronal migration are also reported to localize to the perinuclear region and engage in microtubule stabilization (Sapir et al., 1997; Gleeson et al., 1999; Smith et al., 2000; Tanaka et al., 2004). Although there is no direct evidence that

perinuclear localization of centrobin contributes microtubule stability, it is possible that accumulated centrobin at perinuclear region maintains stable microtubules more efficiently. Another explanation is that the NEK2 phosphorylation affects the oligomerization of centrobin. The oligomerized centrobin may stabilize microtubules by the induction of microtubule bundling. For similar example, the oligomerization state of PRC1 which is known as a midzone-associated and microtubule bundling protein is also regulated by phosphorylation by Cdk (Zhu et al., 2006).

HEF1, a scaffold protein that is localized at focal adhesion and involved in integrin signaling, was reported to inactivate NEK2 kinase activity (Law et al., 1996; Minegishi et al., 1996; Pugacheva and Golemis, 2005). HEF1 controls cell spreading and migration though the regulation of actin filaments (O'Neill et al., 2000). Therefore, it is possible that NEK2-centrobin provides another pathway for HEF1 to regulate cell spreading and migration through the microtubule network. Furthermore, the integrin signaling might regulate microtubule stabilization through an alternative pathway, in addition to Rho-mDia pathway (Palazzo et al., 2004; Wen et al., 2004; Narumiya et al., 2009). Because HEF1 is known as an important player in metastatic cancer, it is also expected that NEK2 functions as downstream of HEF1 in cancer metastasis (O'Neill et al., 2007). It is worth to determine whether HEF1 is a physiological upstream of centrobin or not.

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국문초록

세포골격의 주요구성 성분 중 하나인 미세소관은 튜불린 이합체가 붙었다가 떨어짐을 반복하며 역동적으로 변화하는 구조로 되어있다. 미세소관의 역동성은 다양한 미세소관 결합 단백질들에 의해 조절되는데, 그것들은 대부분 미세소관을 안정화 또는 불안정화하는데 관여한다. centrobin은 새로 생긴 중심립에 존재하며 중심립 복제에 필요한 단백질로 동정되었지만 미세소관 안정화에 관여한다는 일련의 증거들이 보고되었다. 또한, centrobin은 서로 다른 두 인산화효소, NEK2와 PLK1의 기질임이 보고되었다. PLK1에 의한 centrobin의 인산화가 정상적인 방추사의 형성에 중요하다고 알려졌으나, NEK2에 의한 centrobin의 인산화의 기능이 무엇인지에 대해서는 아직 명확히 밝혀지지 않았다. 따라서, 본 연구를 통해 NEK2에 의해 조절되는 centrobin의 기능을 밝히고자 했고, 몇 가지 중요한 생물학적 현상들을 발견하였다.

첫 번째, centrobin이 세포질 내에서 작은 과립형태로 존재하며 간기세포의 미세소관 그물구조와 결합하고 있음을 발견하였다. 나아가 때때로 그것들이 안정화된 미세소관을 따라 크고 길쭉한 형태의 입자를 형성함을 관찰하였다. 이러한 결과는 centrobin이 미세소관의 안정화에 관여함을 뒷받침한다.

두 번째, centrobin이 결여된 세포들은 심각하게 손상된 미세소관을 가지고 있으며, 세포가 바닥에 퍼지거나 이동 또는 증식하는 능력이 감소하게 된다. 반대로, centrobin의 인산화효소로 알려진 NEK2의 경우, 세포

내에서 발현을 감소시켰을 때, 세포의 퍼짐 현상과 이동 또는 증식 능력이 현저히 증가한다. 나아가, centrobin의 발현이 감소되었을 때 간기세포의 미세소관 안정화가 감소하는 반면 NEK2의 발현을 감속시켰을 때는 증가하는 것을 관찰하였다. 이러한 결과는 centrobin이 미세소관 안정화를 통해 세포내의 다양한 활동들에 영향을 미치며 NEK2가 이 과정을 조절할 것 임을 시사한다.

세 번째, 세포 내 또는 세포 외에서의 인산화 반응을 통해 36번째 세린을 포함한 centrobin의 4개의 아미노산 서열이 NEK2에 의한 인산화에 중요함을 확인하였다. NEK2에 의해 인산화가 되지 않는 centrobin을 세포 내에서 안정적으로 발현시켰을 때, 미세소관의 안정화와 세포의 퍼짐 현상과 이동능력이 증가함을 관찰하였다. 그러나 PLK1에 의한 인산화는 이러한 현상에 영향을 미치지 않음을 관찰하였다. 이는 NEK2가 인산화를 통해 간기세포에서의 centrobin의 기능을 특이적으로 조절함을 의미한다.

종합적으로, 이러한 결과들은 NEK2가 centrobin 인산화를 통해 미세소관의 안정화를 조절하며 이것이 세포의 퍼짐이나 이동, 증식 등의 활동에 영향을 미침을 의미한다. 따라서, 본 연구는 centrobin이 새로운 형태의 미세소관 결합 단백질이며 NEK2에 의한 centrobin의 인산화가 간기세포의 미세소관 안정화를 조절하는 중요 경로임을 시사한다.

주요어: 미세소관 안정화, NEK2, centrobin, 인산화, 세포의 퍼짐, 세포의 이동

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